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The Occurrence of Funnel Leaflets and Ring Fasciations in Yellow Lupins

BY

R. F. M. VAN STEVENINCK

(*Crop Research Division, Department of Scientific and Industrial Research, Christchurch, New Zealand*)

With Plates XVI, XVII and three Figures in the Text

ABSTRACT

Funnel leaflets in yellow lupins (*Lupinus luteus* L.) were observed on a mutant, on pea-mosaic infected plants, and on plants treated with 2,4-D. In addition typical ring fasciations were produced on plants treated with 2,4-D. The above phenomena have been discussed in the light of similar morphogenetic effects, reported to be induced in other plants by various growth substances. A possible explanation for the ring-shaped structures has been given by assuming a change in the influence of the apical meristem on developing leaf primordia and by considering the 'physiological field' concept for organ formation respectively.

INTRODUCTION

A LEAF ABNORMALITY of a special type, hereunder described as 'funnel leaflets', was discovered in a mutant of yellow lupins, *Lupinus luteus* L., but later also on plants infected with common pea-mosaic virus.

Similar leaf-abnormalities of a funnel or beaker type have been induced in a wide range of plants as a result of treatment with a variety of chemicals of which 2,4-dichlorophenoxyacetic acid (2,4-D) and tri-iodobenzoic acid (TIBA) were the most active (Kendrick and Middleton, 1954; Krishna Pillai and Chakravarti, 1954; Linser *et al.*, 1953; Price *et al.*, 1950). Conversely funnel leaves of *Codiaeum variegatum f. interruptum* occurring naturally have changed into normal leaves by TIBA treatment (Bünning, 1953, p. 28). It was also possible to induce funnel leaflets in yellow lupins by chemical application, but only a small proportion of the growth-abnormalities were of this type.

It is obvious from this that the morphogenetic organization of primordia responsible for the production of leaves can be interfered with by widely different agents, but that their effects follow similar patterns. With lupins, a mutation and also a virus and chemical agents gave rise to exactly the same type of leaf structure, which has also been found in a wide range of other plants by using the same chemicals. Therefore, this alternative in leaf development, which can be caused by a wide variety of agents, is most likely to arise from a particular change in shape or activity of the leaf primordia. Zimmermann *et al.*, 1944, have reported a similar case in tomatoes. Treatment with certain xylenoxy-acids produced symptoms which resembled very much those caused by virus infection or gene mutation: viz. 'shoe-string' or 'fern-leaf' pattern of tomato leaves. In the discussion an attempt has been made to explain these particular changes in leaf-shape, taking into consideration the

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apical control of development of leaf primordia as has been shown by Wardlaw, 1951, and Sussex, 1951.

An intriguing by-product of the attempt to produce funnel leaflets by chemical means was the induction of ring fasciations, which could be classified into two types: one in which the cylindrical structure carried leaves on petioles, i.e. had shoot properties, and the second in which the cylindrical structure did not carry leaves on petioles except for a crown of leaflets at the very top.

Ring fasciations occur under natural conditions but are very rare (Compton, 1911; White, 1948). However, it has been possible to induce them with chemicals on several plants besides lupins (viz. tomatoes, *Kalenchoë*) (Gorter, 1951; Harder and Oppermann, 1952; van Zeist and Koevoets, 1951). Several ideas have been put forward to explain the initiation of ring fasciations (Compton, 1911; Gorter, 1951; Schoute, 1936; Wardlaw, 1953). In this connexion it was interesting to note that Gorter and Wardlaw on the basis of identical material developed a different view on the basic mechanism underlying this phenomenon. Gorter came to the conclusion that ring fasciations induced by TIBA on tomato would most likely be due to connate growth of several apices while on the other hand Wardlaw supported the view that the same ring fasciations developed from a single apex (true fasciation).

A clear distinction has been made between true fasciation and connation, each being due to an entirely different morphogenetic pathway. Schoute, 1936, using many examples, pointed out the fundamental difference between fasciation and connation (fusion) and quoted Nestler's proposal to exclude connation from the concept of fasciation. The present material, however, indicated that such a sharp distinction between both phenomena could not be made. An explanation has therefore been sought for both phenomena, explaining both types of ring fasciation in lupins as nothing more than a graded difference in response of the apical region towards the chemicals applied.

OBSERVATIONS AND EXPERIMENTS

The mutant

During the 1953/4 season a homozygous line of yellow lupins, *L. luteus* (Weiko II), produced a spontaneous mutant, which appeared identical with an X-ray mutant described by Kress (1953).

The mutant character is typified by an aberrant leaf form (Fig. 1), the leaflets being attached to the petiole by 'sub-petioles' of varying lengths (3–25 mm.), which may be regarded as a midrib on which the lamina is either partially or completely lacking. Microscopical examination revealed a stele with a centric arrangement of vascular elements (Pl. XVI*b*), quite distinct from the bilaterally-symmetrical meristele normally present (Pl. XVI*a*). At the base each leaflet is funnel-shaped for about 3–5 mm. Pl. XVII*a* shows a transverse section of this part of a leaflet. The greatest width of the leaflets is more towards the base than normal, and in some cases the leaflets may be lacking altogether, leaving the midrib only. Still younger leaves, especially those on the laterals, become increasingly complex through the branching of 'sub-petioles', each 'sub-petiole' carrying from 1 to 3 leaflets (Fig. 2*b*). The flowers are much deformed, complicated, and sterile; the anthers are much reduced or absent and the style in some cases is branched or protrudes from the lateral side of the ovary.

Virus infection

Yellow lupin plants infected with pea-mosaic virus (Troll, 1952) at an early stage of development—i.e. before the main inflorescence appears—produce lateral growth throughout the growing-season and continue to do so even when healthy plants have died. The flowers of these plants are much deformed and usually do not produce seed-pods.

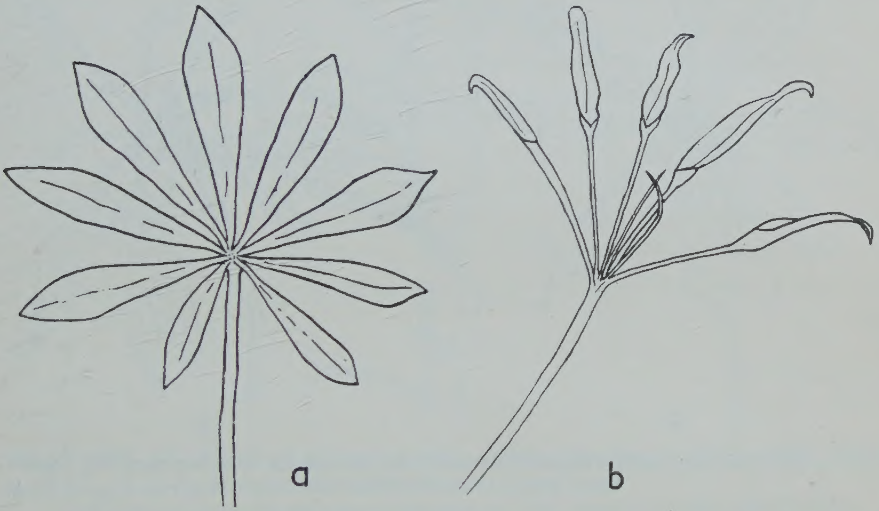

FIG. 1. (*a*) Normal leaf; (*b*) One of the early leaves of the mutant. Some of the leaves discovered on virus-infected plants are completely identical.

An examination of these infected plants led to the discovery of leaves, identical with the ones described for the mutant, at a frequency of one to three per plant and sometimes more, dependent on the amount of total growth produced.

In order to test whether the phenotypic characters of the previously described mutant were in reality due to virus infection, single, typically deformed leaves were taken from the mutant and virus-infected plants and used as infection-sources for aphids (*Myzus persicae*) which were then placed on blue lupins (*L. angustifolius*).

Results showed that in seven out of nine cases pea-mosaic virus could be transmitted from leaves of diseased plants, while in no instance could presence of virus in the mutant be demonstrated.

Chemical treatment

The following experiment was carried out to test the possibility of inducing the funnel leaflets in yellow lupin with chemical treatments. Beta-naphthoxy-acetic acid (β-NOA) and 2,4-dichlorophenoxyacetic acid (2,4-D) were applied as fine mists using solutions of 200, 100, 50, 25, and 10 p.p.m. in water on seedlings (2–3 unfolded leaves plus cotyledons) and on other plants (11–13 unfolded leaves). The applications were given once or twice a week over a period of 3 weeks, or as one single treatment for the whole period.

The action of 2,4-D was much more severe than that of β-NOA but also more effective in bringing about morphogenetic effects. Young plants with a single application of 200 p.p.m. 2,4-D also died. β-NOA had no lethal effect in any of the treatments. A great variety of growth abnormalities was produced by 2,4-D treatments in a single application of 25–200 p.p.m. on all plants and by recurring applications of β-NOA at 200 p.p.m. on young plants only. β-NOA at 100, 50, and 25 p.p.m. was ineffective.

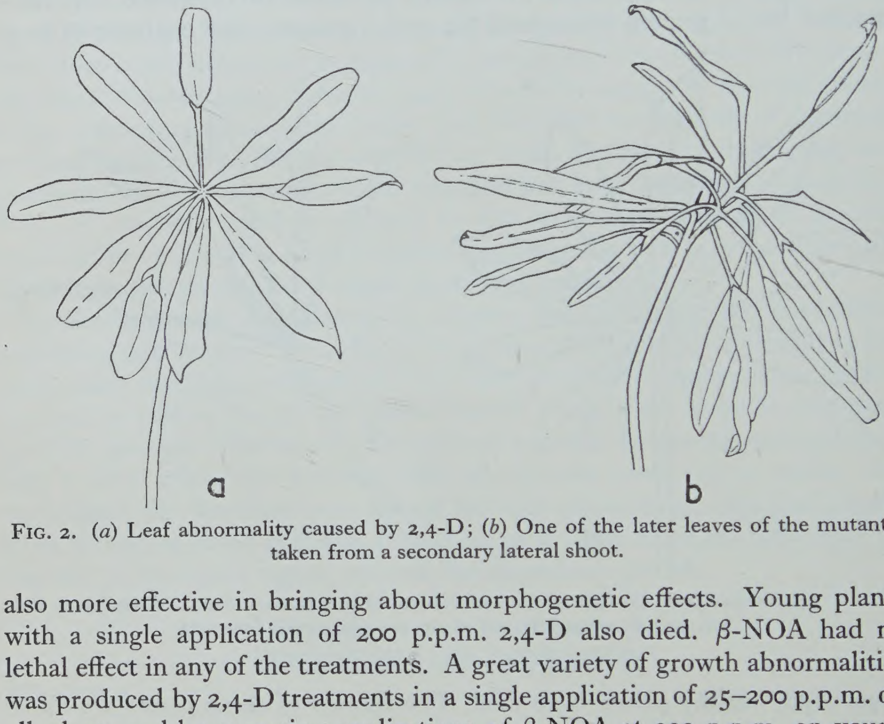

FIG. 2. (*a*) Leaf abnormality caused by 2,4-D; (*b*) One of the later leaves of the mutant, taken from a secondary lateral shoot.

With the 2,4-D applications new leaves appeared at normal growth-rates after severe inhibition of growth, which lasted about 30–40 days, depending on the concentration of the chemical used.

The morphogenetic effects were many and complicated but could be classified into three types:

(*a*) A weak effect, in which single leaflets were involved with the production of funnel leaflets of a similar type to that described for the mutant and virus-induced reaction, with the differences that not all leaflets of a compound leaf were affected and that the remainder of the leaf-blade differed in shape (Fig. 2*a*).

(*b*) A stronger effect, in which the whole compound leaf was transformed into one complete funnel.

(*c*) A still more severe effect induced the apical meristem to form cylindrical structures. In a central position at the base of all these cylindrical structures was an inhibited remnant of the original main shoot. Two types of ring-fasciations could be distinguished which both showed a histological arrangement of tissues very similar to the description of the artificially induced ring fasciations produced by Gorter (1951) in tomatoes or the natural ring fasciations described by Compton (1911) in the mummy-pea; two rings of tissue with vascular elements were present, the vascular elements in the inner ring having an inverse orientation (xylem outside, phloem on the inside). One type of ring fasciation had an apical meristem which produced normal leaves on petioles. This type of ring fasciation was only a temporary condition and soon changed over into a ribbon-shaped structure (Fig. 3). The other type did not have a real apical meristem and consequently did not carry leaves except for a crown of leaflets at the top end (Pl. XVII*b*). With the second type the growth inhibition directly after treatment lasted longer and therefore could be regarded as a more severe effect. The two types of fasciation occurred in a ratio of two to forty-six respectively.

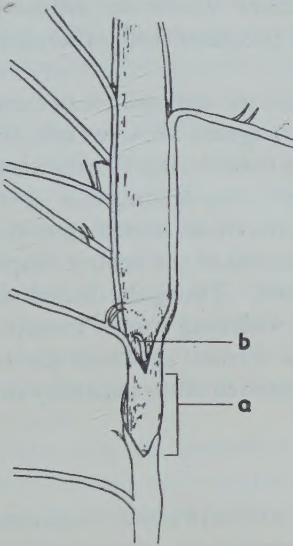

FIG. 3. Ring fasciation caused by 2,4-D treatment. The ring is not complete and the fasciated part carries leafstalks and leaves.

(*a*) Part over which the ring fasciation extends. (*b*) Remnant of the original shoot.

DISCUSSION

Funnel leaves

A wide variety of plants have been reported to produce leaf-abnormalities on application of substances which have a formative effect. It is of particular interest that one special type of these leaf-abnormalities, viz. the funnel or beaker type, seemed to occur repeatedly. In addition it has been shown in lupins that this tendency to produce funnel leaves can result from an inherent condition in the plant as a result of a mutation and that this condition can be duplicated at a relatively late stage by virus infection. It is impossible to even guess any similarities in cellular processes controlled by gene action and those effected by the presence of a virus. However, from other evidence it may be possible to form a picture of the changes in the morphogenetic system of the plant which could be held responsible for the departure from the normal leaf shape. Wardlaw (1951), Sussex (1951), working on *Dryopteris* and potatoes respectively, presented considerable evidence that the flat, dorsiventral, symmetrical structure of a leaf was due to an influence, presumably of a chemical nature, originating from the apical meristematic region. They induced a radially symmetrical structure where an ordinary leaf would have been expected by separating the incipient leaf primordium from the apical region by a tangential incision.

A plausible explanation for the appearance of funnel leaflets must take into account the various causative agents, i.e. virus infection, mutation, and growth-substance treatment. When considering Wardlaw's evidence such an explanation could be proposed upon the assumption of a repression of the apical control over the leaf primordia by an alteration in the metabolism of the component cells, leading to retention of the centric shape of the leaf primordia for a longer period than normal. Thus, the initial suppression of mesophyl growth, passing over into a widening ring of mesophyl, would be followed by a more normal condition as a result of which the leaf becomes dorsiventral. This reaction could be expected to occur generally in many plant species where funnel-leaves are induced.

Ring fasciations

The induction of ring fasciations in tomatoes (Gorter, 1951) and in *Kalanchoë blossfeldiana* (Harder and Oppermann, 1952; van Zeist and Koevoets, 1951) by using TIBA as chemical agent has already been mentioned in the introduction. It appears that whenever a ring fasciation results the original shoot apex or severely inhibited remnant thereof can be found at the bottom of the cavity of the ring-shaped structure, and therefore it is situated just immediately above the place where the ring-shaped primordium has been initiated.

Several theories have been advanced to explain the initiation of ring fasciations (Compton, 1911; Schoute, 1936; Gorter, 1951; Wardlaw, 1953), of which the latter two deal especially with the chemical induction. Two different views will be mentioned here. Gorter on the basis of her evidence came to the conclusion that ring fasciations induced by TIBA would most likely be due to connate growth of several apices. On the other hand Wardlaw (1953) supported the view that a ring fasciation can develop from a single apex. He explains this by assuming a differential effect of TIBA on different regions of the shoot apex, thereby causing cessation of growth in the most distal cells of the apex and permitting slow growth of the subjacent region. The latter would then produce a ring-shaped apex with ultimately a hollow cylindrical shoot.

Ring fasciations in lupins followed two patterns. The first type had shoot properties, i.e. it developed normal leaves on petioles and had an apical meristem and consequently could be classified as a true fasciation. The second and more frequent type did not carry leaves except for a crown of leaflets at its top. Though no grooves or other signs of fusion were present the whole structure appeared as a combination of two or more leaves and therefore could be considered as a connation. Therefore, true fasciation and connation which have been regarded as fundamentally different phenomena (Schoute, 1936) occurred side by side as a result of 2,4-D treatment. Morphologically considered, the difference is a qualitative one, but in fact it may have been caused by a difference in reaction of the apical meristem towards the growth substance or by a difference in amount of growth substance reaching the apical meristem. To account for the two types of ring fasciations an explanation in terms of the physiological field concept (Schoute, 1936; Bünning, 1952: mutual incompatibility of loci of rapid protoplasmatic growth) is proposed.

This concept, that each organ at its initiation and during its development has a certain physiological field of action, would appear to explain adequately: (1) why the tissues between two primordia are inhibited from initiating further organs, (2) why the primordia can be found in a certain position relative to each other, and (3) why they develop as two separate entities, although in equilibrium with each other through the mutual influence of their action fields. On this basis it appears that morphogenetically active substances like 2,4-D can affect the extent and/or intensity of this field of action even so far as to remove its influence entirely. Such an effect could bring about the formation of a ring meristem.

On the other hand where conjoint growth is claimed to have taken place because the organs retain a certain individuality, this could be explained by a weakening of the physiological field of action.

In each case a certain differentiation can take place in the zone producing a ring meristem in spite of the growth-inhibition. The stage of differentiation would then be decisive in determining whether the hollow structure would ultimately have shoot properties (fasciation) or appear as a combination of several leaves (connation).

With ring fasciations in lupins both possibilities occur. Two out of forty-eight could be regarded as belonging to the fasciated type and the remaining ones to the connated type.

Finally it appears that fasciations of organs in general could be explained as the result of a change in their physiological fields. The extent to which this physiological field is affected and the stage of differentiation at that time would decide whether the organs concerned are still distinguishable (connate growth) or whether they have lost their identity completely (fasciation).

SUMMARY

Funnel leaflets in yellow lupins (*L. luteus* L.) were observed on a mutant, on pea-mosaic infected plants, and on plants treated with 2,4-D.

An attempt has been made to explain the general occurrence of leaf malformations of the funnel or beaker type, by assuming a temporary change in

the influence of the apical meristem on developing leaf primordia. As a further result of 2,4-D treatment two types of ring fasciations were produced of which the first type could be regarded as arising from true fasciation and the second one from the connate growth of two or more leaves. The occurrence of both types of ring fasciations has been discussed in connexion with previous theories on the origin of ring fasciations.

An explanation which could account for the two types of fasciations occurring side by side in lupins has been given by assuming a change in the extent and/or intensity of the 'physiological fields' round the growth centres which are involved in the formation of the ring-shaped structures.

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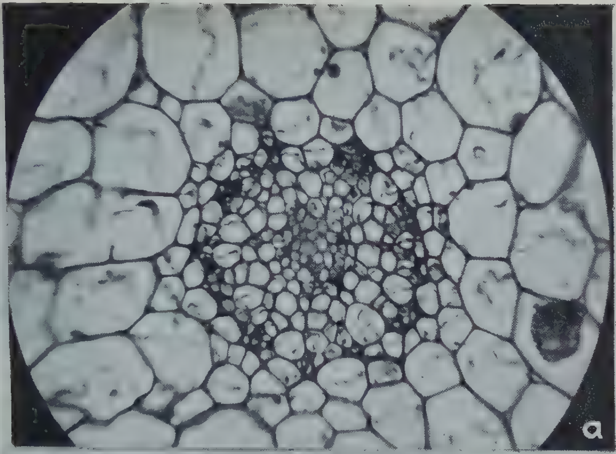
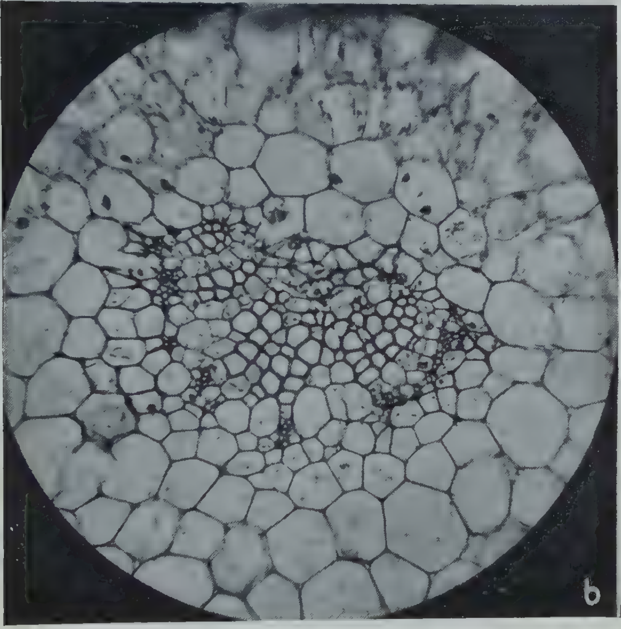
EXPLANATION OF PLATES

Illustrating R. F. M. Van Steveninck's paper on 'The Occurrence of Funnel Leaves and Ring Fasciations in Yellow Lupins'.

PLATE XVI. (*a*) Transverse section of a petiole carrying a funnel leaf. Stele with centric arrangement of vascular elements; (*b*) Transverse section of midrib of a normal leaf.

PLATE XVII. (*a*) Transverse section of the funnel part of a 'funnel leaflet'; (*b*) Complete ring fasciation caused by 2,4-D. The fasciated part does not carry leafstalks and leaves, and is set off with a ring of leaflets at the top. Note the swelling at the location of the inhibited shoot and the small funnel leaves.





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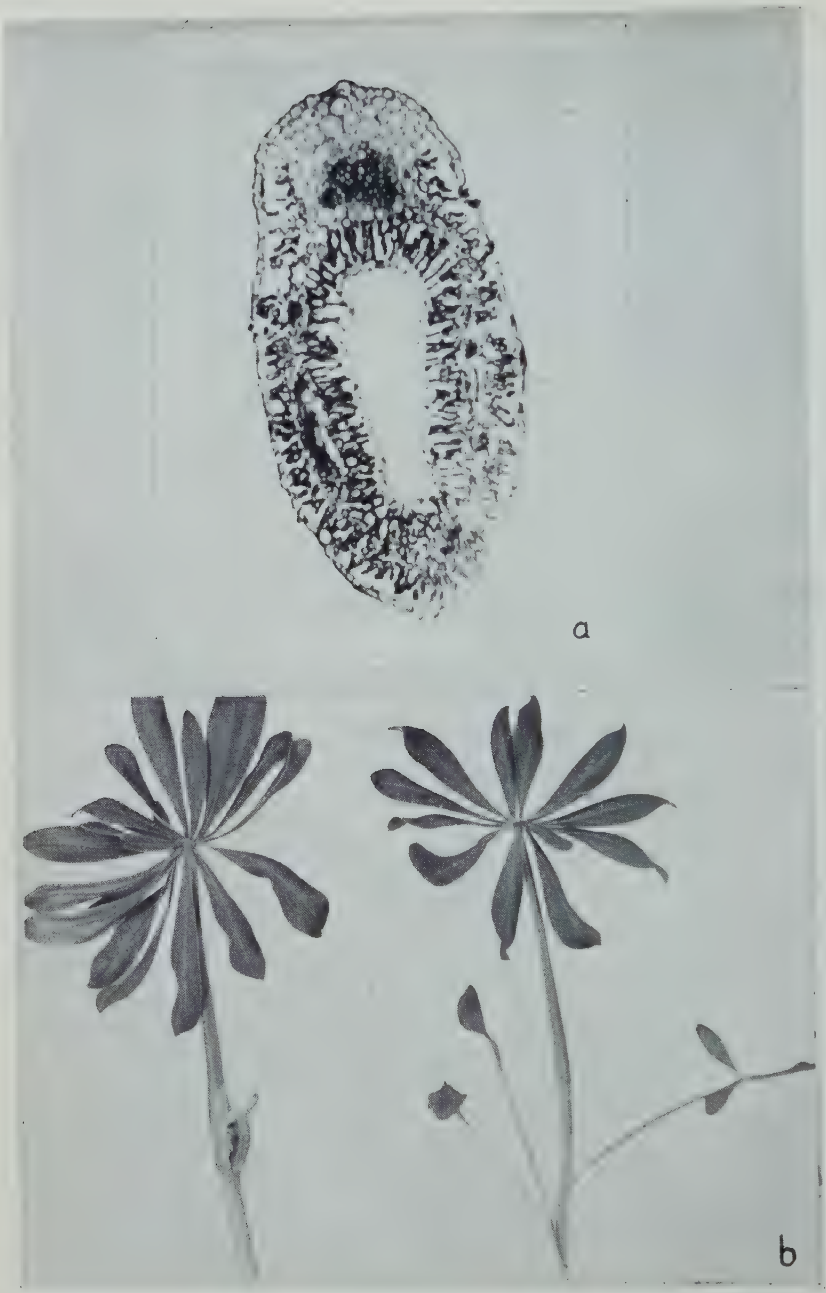

R. F. M. VAN STEVENINCK

Physiological Studies in Plant Nutrition

XVIII. Some Aspects of Nitrogen Metabolism in Barley and other Plants in Relation to Potassium Deficiency

BY

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With two Figures in the Text

ABSTRACT

Barley was grown in water and sand culture using a variety of nutrient solutions, the most important variable being potassium supply. The diamine putrescine, which is not normally found in this plant in appreciable amounts, is present in the potassium-deficient seedling and accumulates during its growth. Maximum accumulation coincides with the development of the severe symptoms associated with advanced deficiency. Putrescine occurs at an earlier stage in potassium-deficient plants whose nitrate and phosphate are supplied as ammonium salts than in those to which the corresponding calcium salts are given. It is found less abundantly in the roots than in the tops. It is absent from protein hydrolysates.

A leaf necrosis characteristic of potassium-deficiency is induced by feeding putrescine to barley having a high potassium status; after prolonged feeding the appearance of such plants suggests severe potassium starvation. The putrescine is slowly utilized, and at the same time an unidentified substance is produced. What seems to be the same substance appears in quantity in high-potassium red clover during the course of rapid utilization of administered putrescine. It also occurs naturally in potassium-deficient red clover. When potassium is supplied to potassium-deficient barley in which putrescine has accumulated, the amine soon disappears. Application of either rubidium or sodium also leads to a reduction in putrescine, though to a lesser extent, rubidium being the more effective element.

Under conditions of extreme potassium-deficiency wheat and red clover also accumulate putrescine. The free amino-acid composition of the wheat then bears a striking resemblance to that of deficient barley.

INTRODUCTION

WHILE it is well known that potassium starvation leads to characteristic deficiency symptoms, almost nothing is definitely known of the functions of this element in metabolism, nor of the immediate causes of the symptoms arising in its absence. The recognition of putrescine as a substance which accumulates characteristically in potassium-deficient barley (Richards and Coleman, 1952), and which appears to be the direct cause of a well-defined necrosis, therefore represents some advance in our knowledge of the etiology of the deficiency disease and of the effects of potassium in

---

[1] This work comprises part of a thesis presented in 1953 for the degree of Ph.D., University of London.

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nitrogen metabolism. In the investigations reported here, and referred to in part by Richards and Coleman (1952), an endeavour was made to determine if the necrotic symptoms of potassium deficiency were in fact directly attributable to the putrescine accumulating under these conditions. Information was sought concerning the ability of other alkali metals (lithium, sodium, and rubidium) to replace this part of the potassium requirement, and a comparison was also made between the effects of potassium starvation on the free amino-acid distribution in barley and the corresponding effects in a few other plant species.

EXPERIMENTAL METHODS

Barley, vars. 'Spratt Archer' and Cambridge No. B2118 (a mildew-resistant variety), wheat, var. 'Atle Spring', flax, var. 'Royal', and red clover (*Trifolium pratense*) were grown in sand culture in the open during the summers of 1952 and 1953. The barley and wheat were sown (9 seeds in a 10-in. pot) during the first week of May and the nutrient solutions applied 5 days later. Two weeks after germination the plants were thinned to 3 per pot. Flax and red clover were sown at the same time at the rate of about 36 seeds per pot and were thinned 3 weeks after germination to 20 and 6 plants respectively per pot. Again the nutrient solutions were applied some days after sowing. In 1952, 149 pots of barley were sown and 13 pots each of wheat, flax, and clover; 6 nutritional treatments were involved. In 1953, 4 treatments were used with a total of 65 pots.

TABLE I

Nutritional Combinations and Pot Numbers in the Sand Culture Experiments

Solution type	1952 Potassium level		Solution type	1953 Potassium level			
	K_1	K_3		K_1	K_3	K_4	K_5
Ca:	31	35	Ca:	18	31	6	10
NH_4:	33	35					
Na:	27	27					

Three main types of solution were employed in 1952: (1) a calcium type in which nitrate and phosphate were given in the form of calcium salts; (2) an ammonium type, with nitrate and phosphate as ammonium salts; and (3) a sodium type, in which the corresponding sodium salts were used. These are standard solution types frequently used in earlier experiments at this Institute. Two levels of potassium were employed with each solution type: K_1, designed to give optimal growth, and K_3, $\frac{1}{9}$ the level of K_1. In 1953 the calcium type solution was used throughout. Barley, flax, and red clover were grown only at the two stated levels of potassium (K_1 and K_3), but with wheat four levels were employed: K_1, K_3, K_4, and K_5; the levels designated by K_4 and K_5 were respectively $\frac{1}{27}$ and $\frac{1}{81}$ of K_1.

Table I shows the various nutritional combinations and the number of pots supplied with each. The symbols used to denote the treatments are composed as follows, after the manner of Richards and Berner (1954). The solution type is indicated first and followed by a colon, after which is shown the potassium level. Thus the symbol $Ca{:}K_3$ refers to the calcium type of solution, with potassium at the K_3 level.

Table II shows the amounts of the actual salts added per pot; to each pot were also added $FeCl_3$ as required and 0·01 g. $MnSO_4.4H_2O$.

For feeding experiments a succession of young barley plants at the same stage of development was required; for this purpose var. Cambridge B2118 was grown in water culture, sowings being made weekly. The nutrient solutions were of the calcium type described above, all salts being supplied in the same relative proportions as in Table II. The solutions were presented, however, at such a strength that the concentration of nitrogen was 100 p.p.m. Potassium was again supplied at the K_1 and K_3 levels, although the significance of these symbols is of course now slightly modified.

TABLE II

Nutritional Scheme for Sand Culture Experiments

1. *Basal nutrients* (g. per pot)

Ca:		NH_4:		Na:	
$Ca(NO_3)_2{\cdot}4H_2O$	12·64	NH_4NO_3	4·00	$NaNO_3$	9·10
$CaH_4(PO_4)_2{\cdot}H_2O$	0·89	$(NH_4)_2HPO_4$	0·05	$Na_2HPO_4{\cdot}12H_2O$	0·18
$CaCl_2{\cdot}6H_2O$	0·50	$NH_4H_2PO_4$	0·77	$NaH_2PO_4{\cdot}2H_2O$	1·04
$MgSO_4{\cdot}7H_2O$	1·25	$CaCl_2{\cdot}6H_2O$	0·50	$CaCl_2{\cdot}6H_2O$	0·50
		$MgSO_4{\cdot}7H_2O$	1·25	$MgSO_4{\cdot}7H_2O$	1·25

2. *Potassium levels* (g. per pot)

	K_1	K_3	K_4	K_5
K_2SO_4	1·85	0·21	0·07	0·023

Method of feeding putrescine to the plants. In a preliminary experiment putrescine was administered to the plants through partially cut leaves and was readily absorbed in this way. Actively growing plants, at the third or fourth leaf stage, were used throughout. In the leaf-feeding experiments with barley the laminae of the last fully expanded leaves were cut transversely, rather more than half-way across, at $\frac{3}{4}$ in. from the base, and then slit for a distance from the end of this cut towards the leaf tip; the midrib was thus included in the resulting flap, which was dipped into the feeding solution contained in a 1×1 in. specimen tube. The flap was passed through a slit in the cork and held in position by a plug of cotton-wool.

Where feeding was to be continued for not more than 3 hours the above method was usually abandoned in favour of feeding the whole tops, either through the roots or through the cut stems. This simplified the work and proved quite as satisfactory as leaf feeding. Root feeding has the disadvantage, so far as metabolic studies in the tops are concerned, that the substance under investigation may have been partly metabolized in the roots and the product translocated to the tops. However, once a metabolic change has been demonstrated by leaf feeding to occur in the tops, root feeding may then provide a convenient means of accumulating larger amounts of an unidentified metabolite preparatory to its isolation and identification.

Feeding solutions were prepared immediately before use and, when necessary, adjusted to pH 5. When an experiment was continued for more than a few hours the solution was renewed frequently. The concentration of the putrescine solution presented to the plants was varied according to the duration of feeding, a range of from 0·1 M to 0·005 M putrescine dihydrochloride being used in order to obtain the desired uptake of amine over feeding periods ranging from a quarter of an hour to several days. The rate of uptake was influenced considerably by atmospheric conditions; when conditions favoured rapid transpiration satisfactory absorption was achieved with feeding solutions of much lower concentration.

Amino-acid analysis. For the free amino-acid analyses, unless otherwise stated, the last fully expanded leaves were used. The extracts were in most cases prepared from fresh material and, after drying, were stored in a desiccator over phosphorus pentoxide until chromatographic analysis could be carried out. In a few instances, however, oven-dried material was extracted. To prepare the extracts, 1·5 g. of fresh plant material was ground in a mortar with 75 per cent. (v/v) aqueous ethanol, the grinding being continued until extraction was complete (15–20 minutes). The insoluble material was separated from the extract by filtration and washed with additional solvent. The combined extract and washings (about 15 ml.) were then evaporated to dryness under reduced pressure, and stored. For analysis, the soluble matter was taken up in 0·2 ml. water, and 10μl. of the resulting solution used for a chromatogram.

Two-dimensional paper chromatograms were prepared by a method essentially that of Dent, Stepka, and Steward (1947), the usual solvents being: (1) phenol saturated with water at room temperature and (2) 'collidine', separated after shaking equal volumes of 2 : 4 : 6 collidine and 2 : 4 lutidine with twice the volume of water. In the later part of the investigation, however, 2 : 4/2 : 5 lutidine (5 volumes) and water (3 volumes) was used in place of 'collidine'. An excellent separation of the basic nitrogenous substances was also obtained with a 5 to 4 mixture of lutidine and water when run at 10° C. After running in these solvents the papers were sprayed with a 0·1 per cent. solution of ninhydrin in ethanol, the colour reactions being hastened by heating at 90° for 7 minutes. Quantitative determinations were not attempted, but an indication of the relative amounts of the various ninhydrin-reacting substances on the chromatograms was obtained from the sizes and colour intensities of the spots, which were graded into nine intensity groups. When a substance characteristically occurred on the chromatograms as a small spot, only its intensity was taken into account.

EXPERIMENTAL RESULTS

Some aspects of the occurrence of putrescine in potassium-deficient barley. Richards and Berner (1954) found putrescine, as an unidentified substance, in very early leaf samples from plants of both the Ca : K_3 and NH_4 : K_3 nutritional treatments, and noted that its amount increased as the leaves turned yellow with age. In the present investigations the putrescine contents of leaves from both these treatments were compared at intervals throughout growth. The amine occurred in the first leaves of the NH_4 : K_3 seedlings, but could not be detected in either the first or second leaves of Ca : K_3 plants. Nevertheless these plants also accumulated putrescine during the following weeks; although the peak concentration was reached some days later than in the NH_4 : K_3 treatment the maximum amount accumulated was approximately the same.

While the high-potassium barley grew well and was of healthy appearance, both the low-potassium treatments resulted in generally poorer plants, with fewer early tillers and prematurely dying leaves. Between the third and fifth leaf stages the lower leaves from the Ca : K_3 treatment became heavily marked with small brown flecks, though this disorder was not evident at any future stage. The NH_4 : K_3 plants did not develop these symptoms, but their growth was even more severely checked than that of the calcium-type plants. At about the sixth-leaf stage sharply defined white areas (a well-known symptom of deficiency) appeared on the leaf blades of plants from both solution types. The putrescine content of these affected leaves was the highest recorded in the investigation. Leaf extracts from the Na : K_3 plants at this time contained a moderate amount of putrescine and a few white areas were present on the leaves. This symptom had been noticed on plants of this type only on one or two previous occasions, and it seems likely that seasonal climatic variation is responsible for the irregularity of its occurrence.

A comparison was made between the relative amounts of putrescine in tops and roots. At a time when there was a moderate amount of the amine in the tops of the NH_4 : K_3 plants, it could barely be detected in the roots. A somewhat similar result was obtained in a later experiment in which high-potassium plants were presented with putrescine round their roots for 2 hours, this treatment being followed by a period of 24 hours in water. They were then extracted, and the putrescine content of the roots proved to be much lower than that of the tops.

In order to discover whether any of the putrescine is bound to protein, the alcohol insoluble residue from potassium-deficient plants has been examined after acid hydrolysis, which was effected by heating the material at 95° C. in a sealed tube for two days with 6N-hydrochloric acid—a treatment to which the amine is stable. Putrescine was not detected in the hydrolysate and it is therefore concluded that it was not present in the leaf protein in a loosely combined form; neither could it be detected among the natural breakdown products of senile leaves from high-potassium plants.

The effect of feeding putrescine to high-potassium plants. The association in time between the development of a well-defined symptom of potassium deficiency and the maximum accumulation of putrescine suggests that this substance may be directly responsible for the leaf injury; a rather similar toxic effect (severe injury and even death of the whole plant) is thought to result from the accumulation of ammonium ions by barley seedlings when grown in very potassium-deficient solutions of the ammonium type. In the following experiment the effects of putrescine on high-potassium plants were examined. The plants used were grown in sand culture in the Ca : K_1 nutrient solution. Putrescine was administered at the third-leaf stage through partially cut leaves, solutions of the dihydrochloride being presented at four concentrations (0·005, 0·015, 0·075, and 0·25 per cent.); three plants were treated with each concentration. The putrescine was readily absorbed in this way.

After some hours the leaves dipping into the stronger solutions developed white margins along the edges of the cuts; this dead tissue was periodically removed to allow absorption to continue. The most suitable concentration proved to be 0·075 per cent., and by the third day sharply defined white areas, simulating extremely closely those which occur normally on potassium-deficient plants, had developed on the distal parts of the young leaves of those tillers receiving this solution. By the fifth day the entire plants were yellowish in colour and growth was apparently checked. When putrescine was supplied simultaneously to several leaves on a single young high-potassium plant, the whole appearance of the plant soon suggested strikingly severe potassium deficiency. In all cases where putrescine was fed to a plant, whether through its leaves or its roots, the characteristic white areas developed; in glasshouse conditions they developed more rapidly than out of doors. Chromatographic examination of an extract of high-potassium leaves in which these symptoms had been so induced showed that their content of putrescine approximated that of potassium-deficient leaves displaying similar symptoms naturally.

High concentrations of some amino-acids have occasionally been reported to be toxic to plants. However, presentation of a number of those which occur commonly, or of ornithine, did not produce the symptoms resulting from putrescine feeding.

Certain changes were found in the free amino-acid contents of barley when putrescine was administered. The amine readily enters the plants, whether presented to the leaves, stems, or roots, and can be detected in the leaves within 30 minutes of presentation to the roots. The effects of putrescine administration were examined chromatographically, and the results are presented here in Fig. 1 in diagrammatic form. In this and similar figures the data are arranged in blocks according to treatment, the particular treatment being indicated at the head by the usual symbols. Each column within a block shows the results from a single plant extract; in most instances of course it represents only a typical result from more than one extract examined. The intensities of the various spots on the chromatograms due to amino-acids and related ninhydrin-reacting substances (derived always from a constant fresh weight of plant material) are represented by the numbers of dots (3–27) entered in the diagram. A blank space indicates that no ninhydrin spot could be detected. Where a spot occurred on a chromatogram but was not identified with certainty a query mark has been inserted.

In these diagrams it should be remembered that the numbers of dots used to indicate the intensity groups form a linear series, whereas the intensity groups themselves, since they depend on visual judgement, presumably represent an exponential series much more nearly than a linear series in terms of colour on the chromatograms, and therefore of quantity of amino-acid present; i.e. as a visual impression the diagrams underrate the colour differences to be seen on the chromatograms between the higher intensity groups relative to those between the low-intensity groups. Bearing this in mind, the method used here to represent roughly the amounts of the substances gives a reasonably accurate picture of their actual variations in so far as any single amino-acid is concerned; but owing to the individual behaviours of the different amino-acids in the ninhydrin reaction, the assessment of the colours does not of course always properly reflect the relative amounts of two different substances.

Block 3 of Fig. 1, Ca : K_1 (2), illustrates the changes found in the free amino-acids during the 48 hours immediately following putrescine feeding. The barley used in this experiment was grown to the third- or fourth-leaf stage in water culture, after which the roots were washed and immersed in 0·1 M putrescine dihydrochloride for 2 hours. They were then removed, again washed and placed in water for the periods stated in Fig. 1. Chromatograms were run from extracts of whole plants, using three plants for each extraction. Immediately after the feeding (0 hours) a moderate amount of putrescine was present. A noticeable reduction occurred during the first 24 hours, and a further diminution during the second day. Some substances, e.g. asparagine, glutamine, glycine, and arginine, were increased (cf. control in Fig. 1), and an unidentified substance (substance '*A*', not normally present) appeared on the chromatograms. In phenol this takes up a similar position to arginine, and in lutidine runs close to lysine. It is of interest that the substance *A* frequently occurs naturally, also in association with putrescine, in low-potassium plants, especially those of the ammonium type.

It appears therefore that putrescine is slowly utilized by barley. This is corroborated by the results of a further experiment in which potassium was supplied to potassium-starved plants already containing the amine. Fig. 1, blocks Ca : K_3 (1) and NH_4 : K_3 (1), illustrates the results of such treatment. The putrescine content, initially high in both the calcium and ammonium type plants, was appreciably diminished by the second day. An additional unidentified substance (substance '*B*', with RF similar to *y*-aminobutyric acid in phenol, and to lysine in lutidine) was now present on the chromatograms. It is noteworthy that a substance chromatographically identical with substance *B* was found in potassium-deficient barley by Richards and Berner (1954); the substance also appeared occasionally in the present series of experiments when barley was fed with putrescine. In an experiment with red clover, to be described more fully later, it was found that this plant

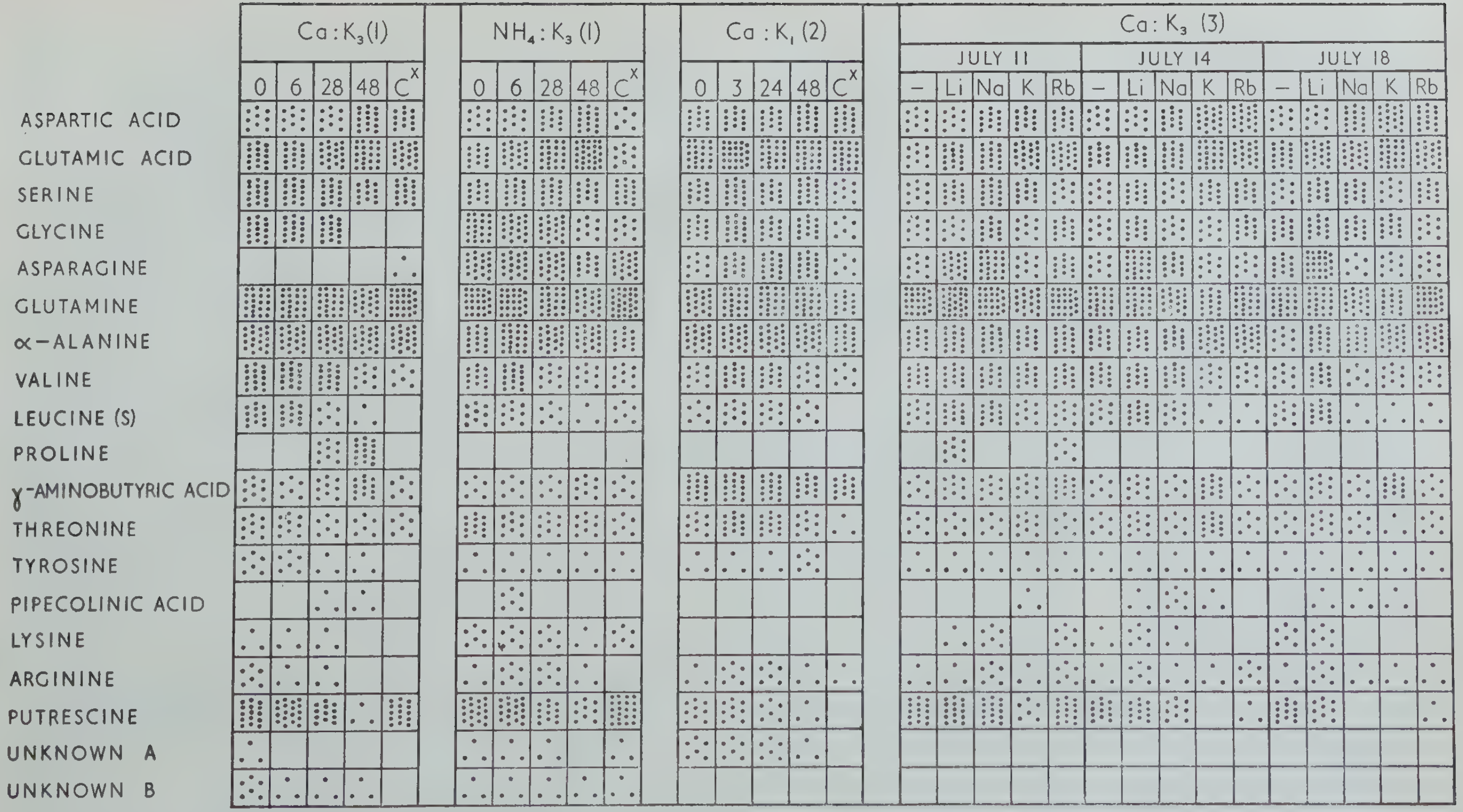

FIG. 1. Diagrammatic representation of spot intensities on paper chromatograms. Notes on the superscripts: (1) indicates K$_3$ plants supplied later with additional potassium, (2) refers to plants kept in distilled water after receiving putrescine through the roots for 2 hours. The numerals under (1) and (2) indicate the number of hours after treatment, and C^x refers to a 48-hours' control. (3) indicates K$_3$ plants supplied on July 9 (9 weeks after sowing) with the alkali metals shown.

attacks putrescine rapidly, giving rise to a considerable quantity of the substance *B*. The inference that the latter is a product of putrescine metabolism, drawn from a relatively slight production during the slow disappearance of the amine in barley, receives considerable support from the much more rapid utilization of putrescine by red clover in association with its accumulation. However, until substances *A* and *B* are identified their place in the metabolism of putrescine cannot be established. Kenten and Mann (1951) have shown that aqueous extracts of pea seedlings oxidize putrescine rapidly; they found that red clover also contains this diamine oxidase activity, though to a lesser degree. On the other hand they did not detect it in barley. An explanation of the difference in putrescine utilization by red clover and barley may well be found in this differential enzyme activity.

Addition of salts of alkali metals to the nutrient solutions of potassium-deficient barley containing putrescine. Richards and Berner (1954) showed that culture solutions containing sodium or rubidium check the accumulation of putrescine in potassium-deficient plants almost as effectively as does an increased potassium supply. In the present experiments alkali metals were given to some potassium-deficient plants at the sixth-leaf stage, when putrescine had already accumulated. Sufficient of either lithium, sodium, potassium, or rubidium was added to the pot to bring its total alkali equivalent up to that of the K_1 level. The symbols Ca : K_3Li, Ca : K_3Na, &c., will be used to indicate these treatments.

Owing to unfavourable climatic conditions the plants grew slowly during May and early June 1953. The first and second leaves from the original Ca : K_3 treatment had become moribund by the end of May, and white areas developed on the blades during the first week of July, about 9 days later than in the previous season. Sulphates of the alkali metals were supplied on July 9. A marked improvement could be seen a few days later in those plants which had been given extra potassium, but not in those given any other supplement. After 9 days the symptoms of potassium deficiency had disappeared from the Ca : K_3K plants and they were growing vigorously, though a purple coloration had developed at the base of the stems, reminiscent of phosphorus deficiency. By this time some improvement had also taken place in both the Ca : K_3Na and Ca : K_3Rb plants. The former were generally greener than their untreated controls, though the colour was not the dark green of high-potassium plants; there was no noticeable increase in their size. The Ca : K_3Rb plants, which were improved in colour and appeared to have many active growing points on July 18, had developed symptoms of rubidium toxicity by July 23. At this time they were erect in habit and generally of a greyish-green colour, but the developing leaves were a pale yellowish-green; some of the internodes of the stems had become considerably swollen.

In the previous season potassium had been added, to three times the K_1 level, to some Ca : K_3 and NH_4 : K_3 plants at the sixth-leaf stage. Twenty-eight hours later a noticeable reduction of putrescine was found to have occurred in plants of both the types, and only a small amount of the amine remained by the second day. In 1953 the first sample was taken 2 days after treatment, and in this case whole tillers were used, since appreciable growth was anticipated; it was hoped by this means to obtain more representative data. Fig. 1, Ca : K_3 (3), shows the chromatographic results from this experiment.

(1) Ca : K_3 (untreated plants). No important changes were evident in the free amino-acid distribution of the control plants during the period over which samples were taken.

(2) Ca : K_3Li treatment. Two days after the addition of lithium a large increase was found in glutamine, moderate increases in glutamic acid and asparagine, and small increases in γ-aminobutyric acid and serine. By July 18 the only important further changes were a slight fall in the putrescine content, a reduction of the excessive glutamine found a week earlier, and an increase in threonine.

(3) Ca : K_3Na treatment. By July 11 aspartic acid and asparagine had increased, but the amide fell later to a level below the control value and the significance of its early rise is doubtful. During the next week there was not only the marked fall in asparagine but a fall also in valine, leucine, and putrescine—which in the sample of July 18 was not detectable; some increase in glutamic acid, and possibly in α-alanine, was also indicated.

(4) Ca : K_3K treatment. After 2 days (July 11) there were increases in the contents of glutamic acid, aspartic acid, γ-aminobutyric acid, and threonine, reductions of glutamine and asparagine and a marked reduction of putrescine. By July 14 these changes were all accentuated, no putrescine at all being detectable; valine and leucine also had diminished in amount, and α-alanine increased. With the probable exception of threonine, the amino-acid contents of these plants remained substantially unchanged between July 14 and 18.

(5) Ca : K_3Rb treatment. By July 11 the only considerable change which was to be maintained later was an increased glutamic acid content. A corresponding increase in aspartic acid had occurred by July 14, as also in α-alanine, but asparagine, valine, leucine, and putrescine had all diminished by varying amounts. The only notable further change during the next 4 days was an increase in the glutamine content; putrescine, though much reduced, could still be detected on July 18.

Certain apparent changes due to these several additions have not been mentioned in the general account given above. The most interesting of these refers to pipecolinic acid, which was evidently produced as a consequence of adding either potassium, sodium, or lithium; rubidium appears, curiously, not to have had a similar effect. The presence of proline too on the 2-day chromatograms from the rubidium and lithium treatments is suggestive, but it is impossible to state whether or not this is significant in relation to the treatments given; Richards and Berner (1954) found this substance to be erratic in its occurrence in barley.

As regards putrescine, therefore, the Ca : K_3K plants showed a marked response to potassium by the second day; the Ca : K_3Rb plants responded rather more slowly, not showing much change until the fifth day. The response to sodium was apparently slower still, but it is possible that it occurred sooner than July 18 (the ninth day). In all these treatments the putrescine accumulated earlier either disappeared altogether, or nearly so. The small reduction found on July 18 in the Ca : K_3Li plants may indicate some response to lithium also, although no improvement was evident in the external appearance of the plants even 3 weeks after the addition of the element.

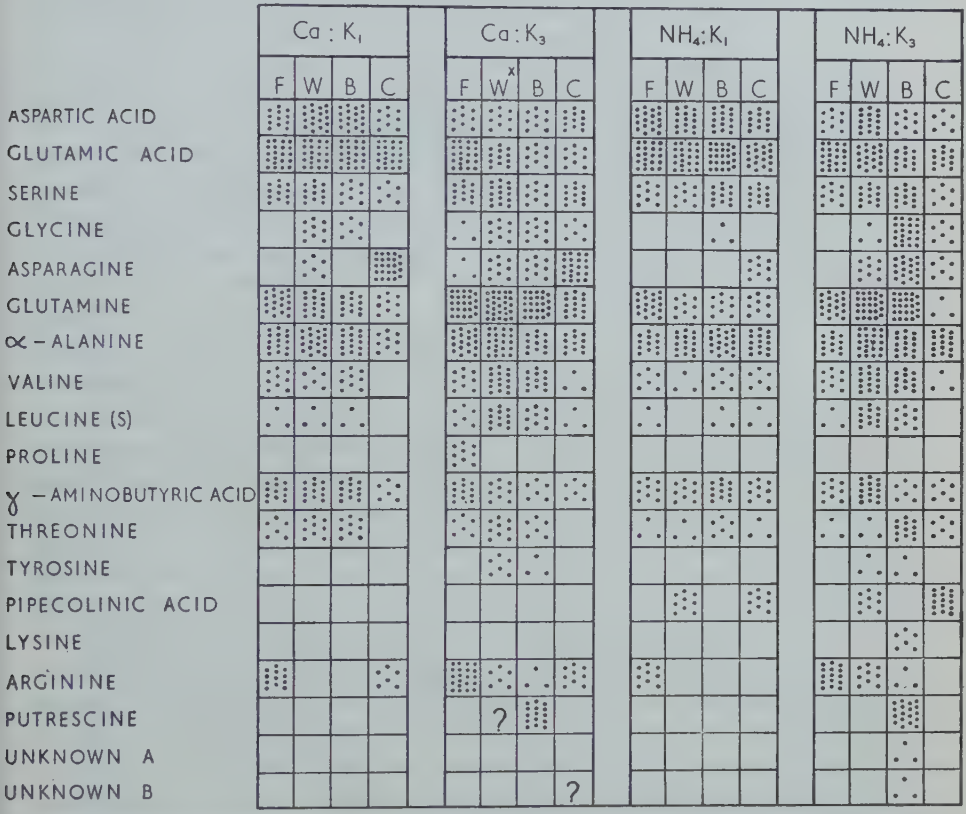

FIG. 2. Diagrammatic representation of spot intensities on paper chromatograms. Notes on the superscripts: F, flax; W, wheat; W^x, Ca:K_5 wheat; B, barley; C, red clover.

While some response in growth to sodium and rubidium accompanied the changes in amino-acid contents, the appearance of the treated plants indicated that these elements can by no means replace potassium fully.

The effect of low-potassium supply on the free amino-acid composition of some other plant species. Fig. 2 shows a comparison between the leaves of flax, wheat, barley, and red clover as regards their free amino-acid contents; all the species were grown in the calcium and ammonium type solutions at two levels of potassium (K_1 and K_3; a chromatogram illustrating the effect of potassium deficiency in the calcium solution in wheat was, however, derived from plants grown at the K_5 level, i.e. one-ninth the K_3 level). The figure presents the data for each species at a time when the plants receiving the low supply showed symptoms of advanced deficiency.

(1) Wheat and barley. A query mark indicates a very weak spot in the position of putrescine on the chromatogram from Ca : K_5 wheat; this was the first occasion on which evidence of putrescine production was detected in wheat. Later it was found in much greater abundance in a plant which had developed white areas on its leaves resembling those characteristic of potassium deficiency in barley.

Visually the NH_4 : K_3 wheat was only slightly affected by the deficiency, but it had much higher contents of asparagine and glutamine, and a lower content of glutamic acid, than the corresponding K_1 plants. These and the other differences to be seen in Fig. 2 due to the low potassium supply are generally consistent with the changes which occur in the amino-acids of barley under similar conditions, and which were described fully by Richards and Berner (1954).

(2) Red clover. The chromatograms derived from red clover differed considerably from those derived from the other species examined. Analyses of these plants at various stages of growth showed that where conditions favoured amide formation, asparagine accumulated rather than glutamine; asparagine production is well known to characterize the nitrogen metabolism of many legumes. The NH_4 : K_1 and NH_4 : K_3 plants, which had lower asparagine contents than usual (Fig. 2; cf. Ca : K_1 and Ca : K_3), were sampled in August 1952, when the nitrogen content of the nutrient solutions would be low, and little nitrogen entering the roots therefore. Unsatisfactory growth was made by the NH_4 : K_3 plants and the symptoms of potassium deficiency were not well defined. However, a ninhydrin positive substance was detected on chromatograms from some Ca : K_3 and NH_4 : K_3 plants in a position indistinguishable from that of the substance B which is found in K_3 barley and was discussed earlier; this substance was also produced abundantly in high-potassium red clover plants artificially fed with putrescine.

In a preliminary feeding experiment the clover was given putrescine through partly cut leaves; these developed brown necrotic areas in the interveinal spaces within 48 hours from the commencement of feeding. This symptom was again strongly reminiscent of a necrosis which characterizes potassium deficiency in the plant. To determine whether the same symptom could be induced by administering putrescine through the roots, roots of some Ca : K_1 plants were immersed in a 0·1 per cent. solution of putrescine dihydrochloride. Small brown dead areas again developed near the leaf margins on the third day. Chromatograms run from leaf extracts of these plants contained a moderate amount of substance *B* (red clover) in addition to putrescine. The root-feeding experiment was repeated, but this time the putrescine was given for 3 hours only, after which the plants were transferred to distilled water for a further 4 hours. Putrescine could not be detected in extracts made at the end of this time, although a moderate amount of substance B (red clover) was present. Hence red clover metabolizes putrescine at a much greater rate than does barley. By making use of this fact and the feeding technique it is hoped to obtain, free from putrescine, sufficient of the substance *B* (red clover) for isolation and identification purposes.

While putrescine was not detected in the potassium-deficient clover grown in 1952, in the following season the Ca : K_3 plants showed very severe symptoms of deficiency and, in addition to the unidentified substance *B* (red clover), contained some putrescine.

(3) Flax. Flax plants grown in either the calcium or the ammonium-type solution had much higher contents of arginine than the species already considered, and under low-potassium conditions had especially large amounts; in fact arginine occurred on some K_3 chromatograms as the most intense spot. Neither putrescine nor the substance *B* was detected.

In all the species examined, potassium deficiency increased the content of arginine. Since barley, wheat, and clover (all of which under these conditions produce either putrescine or the substance *B* which seems to be derived from it) accumulate only small quantities of arginine relative to flax, it appears possible that where putrescine occurs it may be as a breakdown product of this amino-acid. Also, since arginine is a major constituent of many plant proteins (see Lugg, 1949), the rapid proteolysis that takes place in potassium starvation might thus provide an abundant source of amine precursor.

Discussion

The identification of putrescine as a substance accumulating in potassium-deficient barley, and the demonstration that it is directly responsible for a severe necrosis characteristic of advanced potassium deficiency, demands a reconsideration of our knowledge of this disorder. Beginning with the studies of Shih (1934), a considerable amount of work at this Institute has been concerned with differential effects of other nutrient elements in the growth of potassium-deficient plants. Deficiency effects have been investigated over wide ranges of supply of calcium, sodium, and phosphorus. In order to study the effects of potassium supply in low-calcium conditions and the simultaneous absence of sodium, it was necessary to resort to culture solutions supplying the nitrogen and phosphate as ammonium salts. Richards (1941) reported the resulting 'disastrous effects' on low-potassium plants at an early stage, with the loss of many. Other workers (e.g. Turtschin, 1934; Schropp and Arenz, 1939; and Wall, 1940) who used solutions based on ammonium salts had found similar effects when potassium was deficient, and claimed that the disturbance was a toxicity consequent on excess accumulation of ammonium ions within the plant.

Putrescine is now known to be present even at the first leaf stage of such plants (at least in barley), but only in amounts considered to be insufficient to produce the often fatal effects observed. It still seems likely therefore that the deleterious effect of this nutritional treatment on very young plants is largely attributable to ammonium toxicity. Richards (1941) supplied other alkali metals to barley growing at low potassium levels with the object of determining whether they could successfully be substituted for the missing potassium at this early stage. It was found that rubidium if given in small amounts was almost as effective as potassium in preventing the toxic symptoms and in allowing early growth to proceed. Sodium, on the contrary, was quite valueless for this. Later experiments by Richards and Berner (1954) revealed that rubidium and sodium both prevent accumulation of the substance now known to be putrescine, and hence its toxic effects. Putting these facts together it becomes evident that what putrescine is present cannot be of prime importance in the toxicity associated with potassium-deficient *seedlings* growing in solutions containing ammonium salts. The present experiments corroborate the replaceability of potassium by rubidium or sodium in the prevention of putrescine accumulation, rubidium being the more effective.

The toxic effects so far referred to are largely confined to the seedling stage, before potassium deficiency can properly be said to have set in. True deficiency is only reached later, when the potassium available to the plant has been internally 'diluted' by growth. The plants growing in all solution types (not only those containing ammonium salts) now develop necrotic symptoms and the mature leaves die off rapidly, again suggesting toxic effects.

Considerable evidence has been amassed that in these plants inorganic ions of various kinds may accumulate to much higher levels than in high-potassium plants, and that these accumulations may be deleterious. In potassium-deficient barley, sodium, if available, accumulates so greatly as to cause a remarkable degree of succulence (Richards and Shih, 1940). Inorganic phosphate also piles up in supra-normal amounts, and it has been demonstrated (Richards, unpublished work) that this circumstance is detrimental to growth; if the supply of phosphate be reduced when potassium is deficient, the death-rate of leaves is diminished and considerably greater size is attained. One reason for the adverse effect of accumulated phosphate appears to be that it aggravates excessive respiratory losses, abnormally high respiration rates being characteristic of potassium deficiency, at least in barley; these losses also are taking place in a plant whose rate of photosynthesis is considerably subnormal, and whose carbohydrate resources are in consequence already strained. If, at the same potassium level, the phosphate supply be sufficiently reduced the respiration rate falls below that of the normal plant; growth is again reduced and the plant now becomes typically phosphorus starved in appearance. At the optimum phosphate level for growth, then, the low-potassium plant has approximately the same respiration rate (measured in terms of either dry weight or protein nitrogen) as a healthy normal plant, being neither excessive nor depressed (cf. Richards, 1938). Moreover, the necrotic symptoms of typical potassium deficiency are much diminished; growth rate is still not maximal, for the plant will respond to potassium phosphate, but the general appearance is much less unhealthy than if abundant phosphate supply had accompanied the deficiency of potassium.

Clearly then on these grounds excessive phosphate content may well be an important primary factor in the adverse effects associated with potassium deficiency, and may be responsible not only for the inordinate respiratory losses, but also for some of the observed 'toxic effects'. Nevertheless, one piece of experimental evidence favours the view that accumulated phosphate is unlikely to be the primary factor determining putrescine production. This is supplied by those potassium-deficient barley plants which were provided with ample potassium (or rubidium, or sodium) at a time when they had accumulated a considerable putrescine content. Their amine content immediately began to fall, and after a few days had reached a low level. While increased growth accompanied the reduction, it is difficult to believe that the phosphate content could by these means have been reduced sufficiently rapidly to account for the sudden change in metabolism. This fact therefore argues in favour of a more direct effect of the alkali metals themselves.

This paper establishes the presence of putrescine in plant species other than barley. It appears under conditions of low potassium in both wheat and red clover, and may well prove to accompany potassium deficiency in many plants. It has not, however, yet been found in flax. Again, while putrescine has been recovered from deficient red clover, frequently it could not be detected there; whether putrescine was obviously present or not, the unknown substance *B* was nearly always evident. Feeding experiments demonstrated that high-potassium clover converts putrescine rapidly into this same substance; hence there can be little doubt that where the substance occurs naturally in low-potassium clover it has been derived from putrescine.

It is of considerable interest that in barley, under conditions of potassium shortage, a toxic substance such as putrescine, produced in the plant's own metabolism, can accumulate to such an extent that it kills the leaf tissues almost before they have matured. In so doing characteristic irregular areas, from which all leaf pigments have gone, are first developed, i.e. quite white areas. Similar symptoms have also appeared on wheat leaves containing putrescine. Clover on the other hand metabolizes added putrescine rapidly, yielding the basic substance *B*. But this also would appear to be toxic, for where it is present in considerable amounts necrotic areas again develop on the leaves; these, however, are brown in colour owing to the presence of a rather stable brown pigment. The symptoms appearing naturally on leaves of potassium-deficient plants, from which putrescine may be virtually absent, again closely resemble those induced in high-potassium plants by treatment with putrescine. It remains to be discovered whether or not substance *B* is in fact toxic and concerned in this necrotic breakdown. Rather similar brown leaf necroses also characterize potassium deficiency in flax, but in this plant neither putrescine nor the substance *B* has so far been found. Whether in this case arginine accumulation can be responsible for the necrosis has not been determined, but clearly it is desirable to discover the immediate cause of the breakdown.

It is of course true that, as with ammonium toxicity, all these substances which accumulate in association with necrotic symptoms are basic in character (though this has not been completely established in the case of substance *B*), and it might be thought that the basicity is primarily responsible for the breakdown in all the plant types. No systematic investigation of this possibility has yet been attempted, but some very preliminary observations of tissue pH in barley have been made, using the indicator methods of Small (1946), and comparing high- and low-potassium materials collected at a time when severe deficiency symptoms were manifest in the latter. No difference in pH was detectable by the method, and it would appear certain that any difference that may exist must be slight. Claims that the pH of certain tissues is altered slightly by potassium deficiency have been made on a few occasions (cf. Reed and Haas, 1923; and Nightingale, Schermerhorn, and Robbins, 1930), but the changes found were in the direction of increased acidity, not alkalinity. In the feeding experiments reported above, the basic substances were presented as hydrochlorides; it is conceivable that in these circumstances more HCl would be absorbed than substance intended, leading to the development of acidity in the tissues. Nevertheless, plants treated with HCl alone in sufficient amounts to be toxic, and eventually to kill several leaves, did not develop necrotic areas in any way resembling those of potassium deficiency, nor those artificially produced by feeding with putrescine. It seems very probable that something more than pH change is involved. Nothing is definitely known therefore of the cause of the toxicity ascribable to putrescine accumulation.

Basicity, on the other hand, may conceivably help to explain why, in potassium starvation, these particular substances are accumulated. For if alkali metal deficiency shifts the internal balance between inorganic anions and cations in the direction of increased acidity, basic metabolites may become important as compensatory factors, and their metabolic stability increased. This would represent the converse of the well-known balancing of excess inorganic cations with metabolically produced organic acids. On this view the potassium-deficient plant would be compelled to accumulate basic substances, seemingly even to levels at which they become toxic. The apparent relationship, discussed earlier, between free phosphate content and putrescine accumulation might also find an explanation here, for accumulated phosphate must accentuate the inorganic ion unbalance due in the first place to alkali metal deficiency. But the elucidation of these questions must await the results of further research.

ACKNOWLEDGEMENTS

We wish to express our gratitude to Professor F. G. Gregory, F.R.S., for his stimulating interest in the progress of this work. The research was made possible for one of us (R. G. C.) by the award of the Henry George Plimmer Fellowship for 1952 and 1953; he is indebted also to the Agricultural Research Council for a supplementary grant.

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The Relation between the Rate of Nutrient Uptake by Excised Barley Roots and their Content of Sucrose and Reducing Sugars

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ABSTRACT

A re-examination of the results of previous experiments has shown that the positive regression of the rate of nutrient uptake by excised barley roots on sugar content is attributable to the reducing sugar fraction. Partial regression coefficients of the rates of uptake of potassium, nitrogen, and phosphorus on reducing sugar content were all positive; those on sucrose content were either not significant or negative.

These results were confirmed for potassium by experiments in which the sugar content of the roots was varied by varying the nitrogen supply to the plants from which the roots were taken, by shading them or by supplying sucrose to the solution in which the excised roots were held.

PREVIOUS work on nutrient absorption by excised root systems (Humphries, 1951; 1952) has shown that the rates of uptake of nitrogen, phosphorus, and potassium increase with increasing total sugar content of the roots. In these experiments sucrose and reducing sugars were determined separately and summed to give the total sugar content. A further analysis of the results has since been made to find out whether the rates of nutrient uptake are related in the same way, or differently, to each of the two sugar fractions. For this purpose data from the three barley experiments (Humphries, 1951) were combined, and multiple regressions of initial rate of nutrient uptake on the three independent variates, the content of the same nutrient, sucrose content, and reducing sugar content, were calculated for each nutrient. The results are shown in Table I, in comparison with those previously found when the regressions were fitted on total sugar content. The regression coefficients of rate of uptake on reducing sugar content were positive and significant for all three elements; those on sucrose content for potassium and nitrogen were small and not significant, but the regression coefficients of the rate of phosphorus uptake on sucrose content were significantly negative. Evidently, the positive regression of the rates of uptake of nitrogen and phosphorus on total sugar content found previously is wholly attributable to the reducing sugar fraction, while for phosphorus the absence of a significant correlation between rate of uptake and total sugar content is the resultant of correlations of opposite sign with sucrose and reducing sugars. The fractions of the variance of rate of uptake accounted for by the multiple regressions were all significantly increased when sucrose and reducing sugar content were fitted as separate independent variates in

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place of total sugar, and the partial regressions of rates of uptake on nutrient content were all significantly negative; the regression of nitrogen rate on nitrogen content was previously not significant (Table I).

TABLE I

Partial Regression Coefficients ($\times 10^3$) *of Initial Rate of Uptake of Potassium, Nitrogen, or Phosphorus (mg./100 g./hr.) on Content of Potassium, Nitrogen, or Phosphorus respectively* (b_1) *and on Total Sugar Content* (b_2), *(mg. per 100 g.) calculated from the Combined Data from Three Barley Experiments (Humphries, 1951, p. 369)*

	Potassium	Nitrogen	Phosphorus
b_1	−81·9* ±7·9	2·1 ±7·6	−43·2* ±7·1
b_2	11·4* ±3·0	7·5* ±1·0	−0·1 ±0·2
Per cent. of variance accounted for by regression	84	68	50

Partial Regression Coefficients ($\times 10^3$) *of Initial Rate of Uptake of Potassium, Nitrogen, or Phosphorus on Content of Potassium, Nitrogen, or Phosphorus respectively* (b_3), *Reducing Sugar Content* (b_4) *and Sucrose* (b_5)

	Potassium	Nitrogen	Phosphorus
b_3	−59·0* ±8·4	−25·4* ±9·3	−41·9* ±6·2
b_4	19·0* ±2·9	12·5* ±1·5	0·9* ±0·3
b_5	2·0 ±1·8	1·4 ±1·7	−0·7* ±0·3
Per cent. of variance accounted for by regression	89	78	62

These results suggest that the reducing sugar component of the total sugar in the roots is concerned in the mechanism of nutrient uptake—the higher the reducing sugar content the greater the rate of uptake—while sucrose in the roots may have no effect and may even impede the uptake of phosphorus. To obtain further evidence in support of these conclusions, two experiments were done in which the sugar content of the roots was varied by adding sucrose to the nutrient solution from which the excised roots were absorbing, by growing the plants with varied nitrogen supply, or by shading them before the roots were excised. In one of these experiments glucose and fructose in the root extracts were separated by paper chromatography and estimated independently to see if rate of nutrient uptake was specifically influenced by these sugars, but the results were not interpretable with certainty and will not be discussed here. Rates of uptake of potassium, nitrogen, and phosphorus were determined, but only potassium gave significant results, and those for the other elements will not be considered in detail.

ARRANGEMENT OF EXPERIMENTS

The experiments were of factorial design, comparing all combinations of low nitrogen *v.* high nitrogen supply (N) to the growing plants, no shading *v.* shading (S) before the roots were excised, and no sucrose *v.* sucrose (C) added to the nutrient solution in which the excised roots were placed. In each experiment the eight treatment combinations were tested in duplicate.

The general procedure was the same as previously described (Humphries, loc. cit.). Barley plants were grown either in half-strength Hoagland solution or in a solution deficient in nitrogen (containing 1/30 of the amount of nitrogen). The solutions were not renewed during the 6 weeks' growth period. Duplicate cultures were set up with an interval of a week between. Four days before the experiment began one-half of the plants in each treatment were put in the dark to reduce the carbohydrate content of the roots. At the beginning of the experimental period samples of the plants were taken and the roots were excised, washed in distilled water, centrifuged to remove adhering moisture, and weighed. An initial sample was taken for analysis and another sample was transferred to vessels through which aerated nutrient solutions flowed at constant rate. The vessels were held in a thermostat at 25° C. After a period of 8 hours' absorption from the flowing nutrient solution (full-strength Hoagland solution with or without 2 per cent. sucrose), the roots were washed in distilled water, centrifuged, and weighed. Approximately half of each sample was refluxed in boiling alcohol for subsequent carbohydrate determinations and the remaining portion was oven-dried for determination of dry weight and for analysis. The apparatus was thoroughly cleaned by means of detergents and distilled water before the commencement of each experiment but otherwise no attempt was made at sterilization.

In these experiments there were only two sampling times, at 0 and 8 hours, so that the procedure followed in previous experiments (Humphries, 1951; 1952) of estimating rates of uptake at particular times by the slope of the quadratic curve fitted to the nutrient contents for the six sampling times was inapplicable. Instead, the rates of uptake used were the increases over the 8-hour period, and the nutrient and sugar contents were the means of those found at 0 and 8 hours.

RESULTS

The mean effects of the three factors, supply of sucrose to the excised roots, shading, and nitrogen supply to the growing plants, on the sugar and nutrient contents of the roots, and on the rates of nutrient uptake are summarized in Table II.

In Exp. 1 the roots had more than twice the sucrose content of those used in Exp. 2, but the mean reducing sugar contents were identical, and the mean potassium, nitrogen, and phosphorus contents were similar, in the two experiments.

Addition of sucrose to the nutrient medium (C) had nearly equal effects on the sugar content of the roots in both experiments, causing a greater increase in sucrose than in reducing sugars. As this factor operated only during the experimental period, it had no effects at 0 hour, so the mean effects on potassium, nitrogen, and phosphorus contents shown in Table II represent half the effects found at 8 hour, and are therefore identical with half the mean rate of uptake of phosphorus, potassium, and nitrogen.

In Exp. 1 shading (S) caused large and nearly equal decreases in sucrose and reducing sugar contents; in Exp. 2 it had a similar effect on reducing sugars, but the reduction in sucrose content was only about half that in Exp. 1. It had negligible effects on mean nutrient content.

Additional nitrogen (N) caused a very large reduction in sucrose in Exp. 1 but increased the reducing sugar content. In Exp. 2 it had no effect on sucrose content, but increased the reducing sugar content even more than in Exp. 1. It increased the nitrogen content of the roots and depressed the potassium content in both experiments. It also reduced the phosphate content in Exp. 1 but not in Exp. 2. In both experiments the decreases in reducing sugar content caused by shading were greater in plants that received the higher nitrogen supply; this was the only interaction of any consequence.

TABLE II

Mean Effects of Sucrose (C), Shading (S) and Nitrogen (N) on the Composition of the Roots and on the Rates of Nutrient Uptake

	Mean content, mg./100 g.					Mean rate of uptake, mg./100 g./8 hr.		
Exp. 1	Sucrose	Reducing sugars	Potassium	Nitrogen	Phosphorus	Potassium	Nitrogen	Phosphorus
Mean	547	340	301	139	38	134	53	3
Effects of:								
C	120	64	21	4	2	41	8	4
S	−257	−222	2	−12	−3	−54	−9	−2
N	−448	188	−95	58	−17	110	1	5
S.E.	79·1	24·5	10·1	5·1	2·1	22·4	5·9	2·1
Exp. 2								
Mean	220	340	305	163	45	115	42	−1
Effects of:								
C	108	77	45	12	3	91	24	6
S	−107	−184	17	1	2	−6	0	2
N	2	417	−70	84	2	149	24	6
S.E.	38·9	54·1	8·9	3·8	1·0	20·7	4·2	2·5

Associated with these changes in composition of the roots, there were large changes in the rate of potassium uptake (Table II). The mean rate was similar in the two experiments; in both it was increased by supplying sucrose to the excised roots, it was decreased by shading (but not significantly in Exp. 2), and it was much greater in roots from plants receiving the higher nitrogen supply. [There was a similar pattern in the responses of the rates of uptake of nitrogen and phosphorus, but the treatment effects were more variable and in Exp. 1 they were not significant. As no consistent correlations could be established between rates of uptake of nitrogen or phosphorus and sugar content, they will not be discussed further.]

Two of the three factors tested, supplying sucrose and shading, altered both sugar fractions in the same sense; only nitrogen supply produced differential effects and so provided a basis for discriminating qualitatively between the effects of varying sucrose or reducing sugar contents. Higher nitrogen supply increased the rate of potassium uptake and the reducing sugar content of the roots, but decreased the sucrose content, suggesting that the reducing sugar fraction is responsible for the correlation of rate of uptake with sugar content. The same conclusion follows from a comparison of the two experiments; the mean rates of potassium uptake were similar, in agreement with the equal mean reducing sugar contents, and in spite of the much lower mean sucrose content in Exp. 2 than in Exp. 1. When the mean rates of potassium uptake for each of the eight treatment combinations are plotted against mean sugar content (Fig. 1), there is an obvious tendency for the rate of uptake to increase with increase in reducing sugar content over the whole range, but no simple relation between rates of uptake and sucrose is apparent.

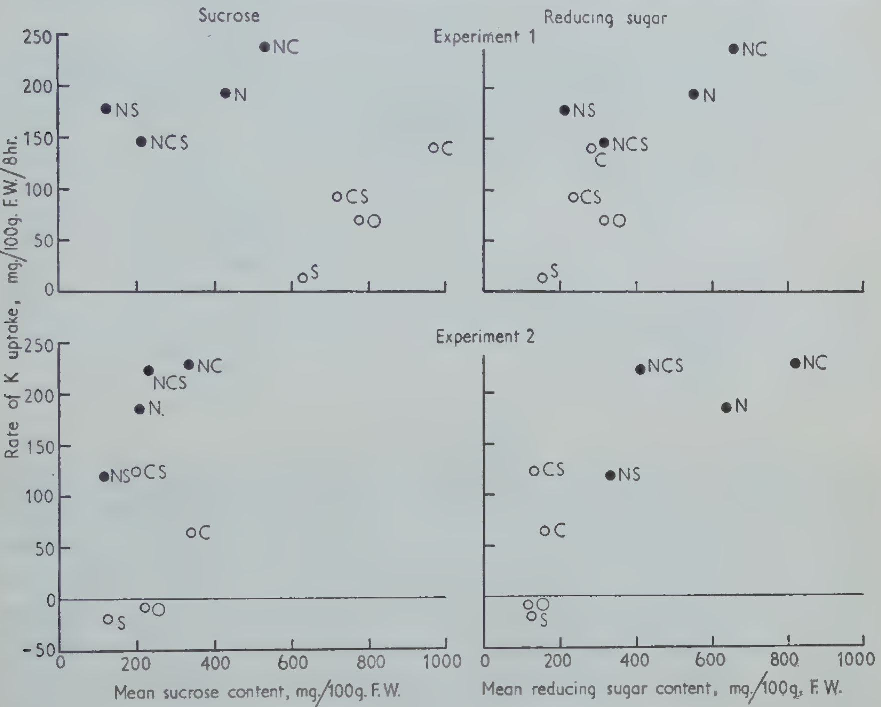

FIG. 1. The relationship between rate of potassium uptake by excised barley roots and their sucrose and reducing sugar content.

Multiple regressions of rate of potassium uptake on the mean contents of potassium, sucrose, and reducing sugars were calculated for each experiment from the treatment means that are plotted in Fig. 1. The regression coefficients on reducing sugar content (b_2, Table III) were positive and significant in both experiments; those on sucrose (b_3) were both negative, but only that for Exp. 2 was significant. Contrary to the results found in previous experiments (Table I) the regression coefficients on potassium content (b_1, Table III) were both positive, and that for Exp. 2 was significant. The reason for this discrepancy is not clear; it may be partly due to the fact that the effects of the sucrose treatment (C) on mean rate of potassium uptake and on mean potassium content were not independent; in fact they were identical, apart from a factor of 2, because they were both determined by the effects on potassium content at the end of the experimental period (see p. 413).

TABLE III

Partial Regression Coefficients ($\times 10^2$) *of Rate of Potassium Uptake* (*mg./100 g./8 hr.*) *on Potassium Content* (b_1), *Reducing Sugar Content* (b_2) *and Sucrose Content* (b_3) (*mg./100 g.*)

	Exp. 1	Exp. 2
b_1	54·5 ±77·6	265·9* ±66·0
b_2	38·4* ±12·7	76·5* ±12·8
b_3	−17·1 ±12·4	−82·8* ±27·0
Per cent. of variance between treatments accounted for by regression.	49	89

DISCUSSION

The results of these and the previous experiments show unequivocally that conditions that induce high reducing sugar contents in excised barley roots also induce high rates of potassium uptake. In the same conditions the sucrose content may also be high, but the regression analysis shows that when the correlation with reducing sugars is eliminated, high sucrose contents tend to be associated with low rates of potassium uptake. There is evidence that similar relationships hold for the rates of uptake of nitrogen and phosphorus.

Variation in nitrogen supply to the growing plants must affect other properties of the roots as well as their sugar content, and it could be argued that some of these, rather than the correlated sugar changes, cause the changes in rate of potassium uptake. Shading the plants or feeding sucrose to the excised roots presumably influences the carbohydrates of the roots more directly, and as their effects on rate of potassium uptake can be explained consistently with those of varying nitrogen supply in terms of the changes in reducing sugar content, it is reasonable to conclude that there is a fairly close metabolic connexion between reducing sugars and nutrient uptake.

Surrounding an excised root system with solutions of varying osmotic pressure may cause both a variation in the amount of liquid exuded by the vessels of the cut ends and the osmotic pressure of the exuded liquid (e.g. Arisz *et al.*, 1951). It is clear that in the present experiments it is the net uptake of each element that has been measured, i.e. the difference between the total amount absorbed and the amount exuded, if any. If the presence of external sucrose by virtue of the increased osmotic pressure causes a greater exudation of the solutes under investigation than in the absence of sucrose, the increased rate of uptake due to greater sugar content of the roots will have been underestimated.

Whether the dependence of uptake of potassium on reducing sugar content is to be related to the general metabolism of the cell or is more specific and is concerned with the carrier system as suggested by Roberts *et al.* (1949) in the case of *Escherichia coli* it is not possible at the moment to say.

From the practical point of view the relationship between rate of uptake and reducing sugars may be of some importance because, as the present experiments indicate, nitrogen supply can influence the relative proportion of reducing sugars and sucrose present in root cells. It is known that variation in other elements such as potassium and phosphorus may cause alteration in the ratio of sucrose and reducing sugars in plant tissues.

ACKNOWLEDGEMENT

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On Two Compressed Palaeozoic Axes: *Stigmaria ficoides* in the *Gymnostrobus* condition and *Vertebraria indica*

BY

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With Plate XVIII and four Figures in the Text

ABSTRACT

The possible structure of *Vertebraria* is considered in the light of evidence based on *Stigmaria—Gymnostrobus salisburyi*.

INTRODUCTION

THE paper describes two Palaeozoic axes; one, *Stigmaria*, is one of the commonest fossils in the Northern Hemisphere and the other, *Vertebraria*, one of the commonest in the Southern Hemisphere and India. The structure of *Stigmaria* is well known from petrifactions which serve to explain all the features of the casts and compressions, but *Vertebraria* is not yet known petrified and its structure is still a matter of conjecture. One form of preservation of *Stigmaria*, called *Gymnostrobus salisburyi*, has, however, been misunderstood and regarded as a cone and this is described here; part of its interest lies in the light it throws on *Vertebraria*.

STIGMARIA IN *GYMNOSTROBUS* CONDITION

The principal specimen discussed here is a slightly compressed sandstone cast labelled 'No. 37, Sandstone showing *Stigmaria*. Coal Measures, Yell Co., Arkansas, Ward's Natural Science Establishment, Rochester, N.Y.' It is in the Hunterian Museum, Glasgow University (No. Pb. 536). Its outer surface shows the usual rootlet scars (Pl. XVIII, Fig. 1) and its interior exposed by a longitudinal split shows the woody core (Text-fig. 1 A; Pl. XVIII, Figs. 2, 3). The whole specimen has compressed to about half (11 cm. wide, up to 6·5 cm. thick), and inside the xylem core is a pith cast also compressed to about half (2 cm. × 0·9 cm.). The entire core is slightly displaced and the whole fossil is somewhat obliquely flattened. There is a groove on one side, presumably the upper surface when in its original position in the sediment, opposite and equal to the xylem core in width (Text-fig. 1 C; Pl. XVIII, Fig. 1).

The pith cast shows a pattern of raised sandstone ridges (Pl. XVIII, Fig. 2) separated by an elongated network of coaly lines (Pl. XVIII, Fig. 3). Towards the sides this pattern changes and instead of the raised ridges there are rectangles of sandstone separated by thin films of coal, the rectangles being about 5–6 mm. wide and 7 mm. high; their thickness is minute. These rectangles

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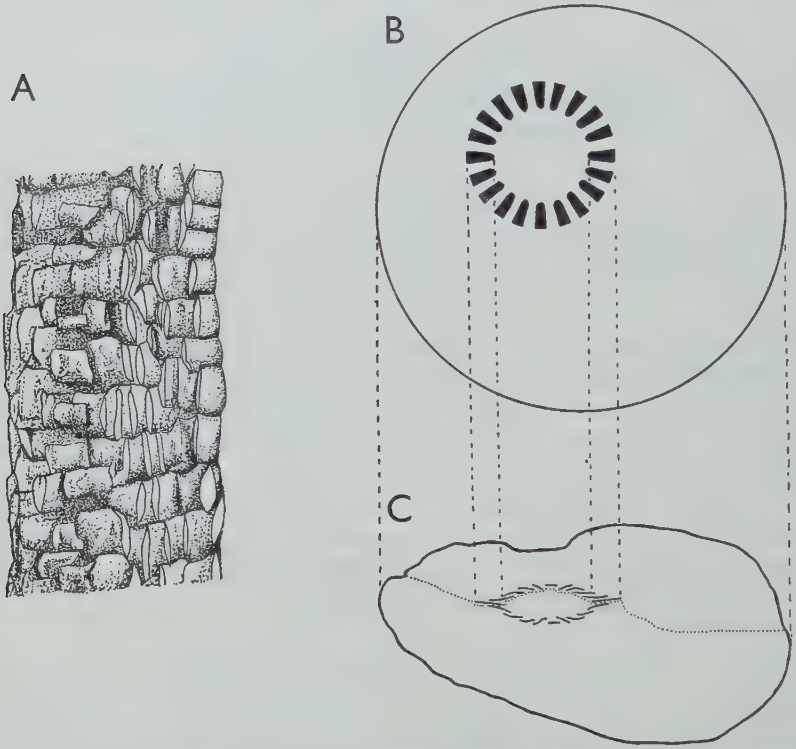
are finely striated in the direction of the long axis of the fossil; the striations look like the imprint of tracheids but the fine details are ill preserved.

A second specimen in the Hunterian Museum (Pb 2364) is from Dollar, Scotland, and consists of an isolated woody core preserved in the same way. It bears the label *Gymnostrobus salisburyi* (the specimen was examined by Dr. W. G. Chaloner who recognized that it was not a cone). The stele is more flattened and the pattern on the pith cast is less clear, but the fine details are better preserved. Dry celloidin pulls from the rectangles yielded typical *Stigmaria* tracheids in abundance.



TEXT-FIG. 1. *Stigmaria ficoides*. A, counterpart of Pl. XVIII, Figs. 2, 3, showing matrix-filled rays, × 1. B, supposed form of the specimen (with displaced stele) just before compression. C, the same after compression and collapse of xylem. The line of cleavage is indicated by dots, × ½.

Walton (1936) has proposed a theory of the alteration of plant form in compression based on experiment and on observation of fossils. According to this theory the mud underneath a specimen took the form of the undersurface of the specimen and the upper part sank downwards. All movement in compression is collapse in a downward direction; there is no appreciable lateral expansion at all. On this theory I picture the preservation of the American specimen as follows:

A broken piece of *Stigmaria* was buried in sandy mud. Its cortex was perhaps a little distorted but it was rigid enough to impress its shape on the mud beneath it, and then the soft tissues between the xylem and the cortex rotted, admitting mud which flowed into the pith cavity and large rays. After a while the outer cortex and the xylem both rotted and compressed under the weight of the sediments above. The mud compressed to about half but the xylem and outer cortex crushed to a minute fraction, a mere film of coal. The belated collapse of the xylem caused a groove on the upper surface (Professor Walton tells me that such a groove, always on the upper surface, is commonly seen in *Stigmaria*, as, for example, in the Fossil Grove, Victoria Park, Glasgow). The mud occupying the rays suffered some distortion as a result of the collapse of the adjacent xylem, particularly so in the rays lying at 45° to the vertical, but their relative positions remain recognizable. The plane of cleavage passes through the coaly films of compressed xylem or along their contact with the rays so that the rays form conspicuous rectangular ridges at slightly different levels along the sides of the xylem core.

The nature of this specimen as *Stigmaria* has never been doubted, but its xylem core, in isolation, looks exactly like *Gymnostrobus salisburyi* (Lesquereux) Bureau (see Bureau, 1914, Pl. 38). This agreement is in all gross features and extends to the cross striation of the lateral rectangles (rays) caused by the tracheids.

The history of the attribution of such xylem cores to cones is as follows:

Feistmantel, 1875, Pl. 45, Fig. 2, figured a xylem core of this form as *Lepidostrobus*.

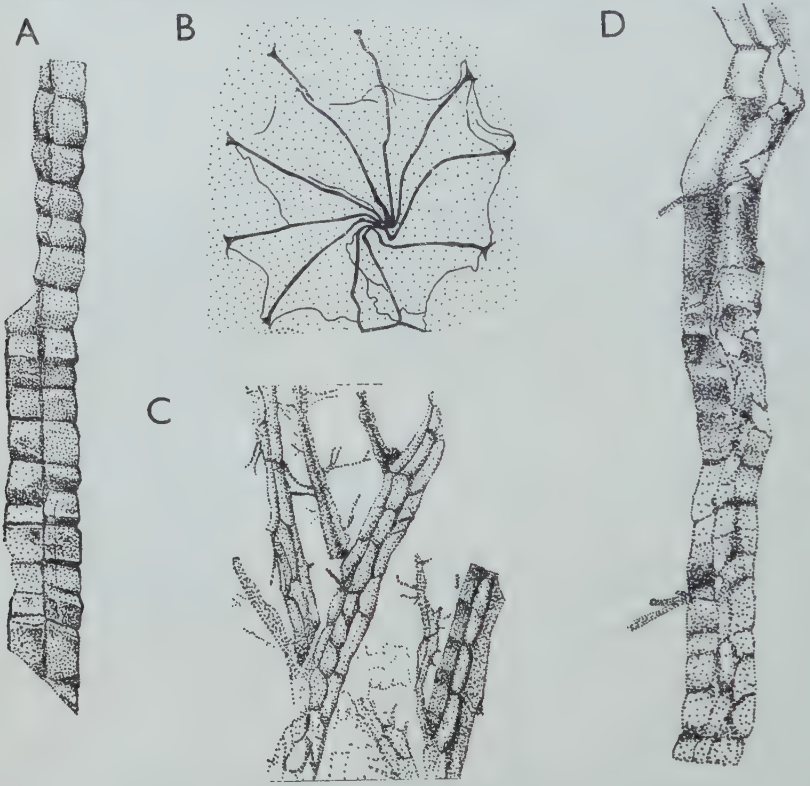
Lesquereux, 1879–80, p. 443, Pl. 69 and pp. 454, 455, 458, described several *Stigmaria* xylem cores as cones under the names *Lepidostrobus* (*Macrocystis*) *salisburyi* sp. nov., *Lepidophyllum truncatum* sp. nov., *Lepidocystis pectinatus*, and *L. lineatus* sp. nov. Later (1884, p. 784, Pl. 108, Fig. 1) he included a true cone, with megaspores 1 mm. wide, under the name *Lepidostrobus* (*Macrocystis*) *truncatus*.

Kidston (1902) described a Scottish specimen (as *Stigmaria*) with a xylem core which Jongmanns (1930, p. 379) pointed out was exactly like *Gymnostrobus*. Accordingly Jongmanns attributed *Gymnostrobus* to *Stigmaria* but he gave no details. Bureau, 1914, pp. 165–7, Pl. 38, Figs. 1, 1 A, 2, 2 A, figured two French specimens of identical xylem cores as *Gymnostrobus salisburyi* and referred Lesquereux's *Lepidostrobus* (*Macrocystis*) *salisburyi* and *L. truncatus* to the same species. He discussed them in detail and refers to megaspores 1 mm. wide, but these do not appear to have been seen by him and may arise from the earlier confusion with cones. Bell (1944, p. 95, Pl. 61, Fig. 2, Pl. 62, Fig. 4) figured two large isolated xylem cores from Canada as *Gymnostrobus salisburyi*.

VERTEBRARIA

The group of axes called *Vertebraria indica* Royle is very widespread in the Lower Gondwana (Permo-Carboniferous) '*Glossopteris*' floras. It is widely held to be the stem or rhizome of *Glossopteris*. It has been discussed by many authors, particularly Zeiller (1896, 1902), Oldham (1897), Seward (1910), and more recently by Walton and Wilson (1932) who discuss the various interpretations of the fossil and give references to still other interpretations, now abandoned.

The fossil is preserved in one of two ways. It may stand vertically in the rock when (in section) it appears as about five to ten spokes of carbon radiating from a centre (Text-fig. 2 B). More commonly it lies horizontally, when it is seen as an elongate organ usually showing two longitudinal series of rectangular areas, but often three series and sometimes more. Text-fig. 2 A shows the appearance of the type (Royle, 1833, Pl. 2, Fig. 1), and Text-fig. 2 C, redrawn from Bunbury's specimen (1861), shows three series. A rather small specimen described here (Text-fig. 2 D) shows two series in one part, three in another. Walton and Wilson (1932) showed that the thin coaly layer on the surface of these rectangular areas consists of secondary xylem and they put forward the theory that the broad rays had rotted and were filled with mud



TEXT-FIG. 2. *Vertebraria indica.* A, Royle's type specimen redrawn, showing the two series of slightly raised rectangular areas. At the left top the plane of cleavage passes along a xylem ray going obliquely downwards. V. 4189, × ½. B, end of vertical axis showing radiating plates of coal, redrawn from Walton and Wilson, 1932, p. 204, Text-fig. 3 A, × 3. C, part of specimen figured by Bunbury, 1861, Pl. XI, Fig. 3, showing three series of rectangles, redrawn from V. 19653, × ½. D, specimen described in this paper from a drilling core from the Mhukuru Coalfield, Tanganyika (DH 401/4757). Two roots are seen apparently attached. × 2.

while the coal films represent the compressed secondary xylem radiating from the centre: this theory is strongly supported here. (A few specimens are believed by some authors to show the tissues outside the wood, but as the present material throws no light on the outer layers they are not discussed here.)

The present specimen was found in a richly fossiliferous drilling core from the Mhukuru Coalfield, Tanganyika (No. 401/4747 and 4757, see Harkin, 1953, p. 28), which was handed to me by Professor Walton for investigation. Though small, it is excellently preserved. 'Dry celloidin pulls' were made from it by covering it with cellulose nitrate in amyl acetate and these when dry were simply pulled off and mounted in Canada balsam.

Four different kinds of tissue were seen in different parts of the pulls:

1. *Narrow spiral tracheids* (protoxylem) associated with slightly wider scalariform tracheids (Text-fig. 3 A; Pl. XVIII, Fig. 6). These spiral tracheids were found near the periphery of the specimen but there is nothing else to show their relation to the secondary xylem. They might belong to a root stele and have no relation to the main *Vertebraria.* Walton and Wilson obtained similar protoxylem which showed 'annular or scalariform' thickenings.

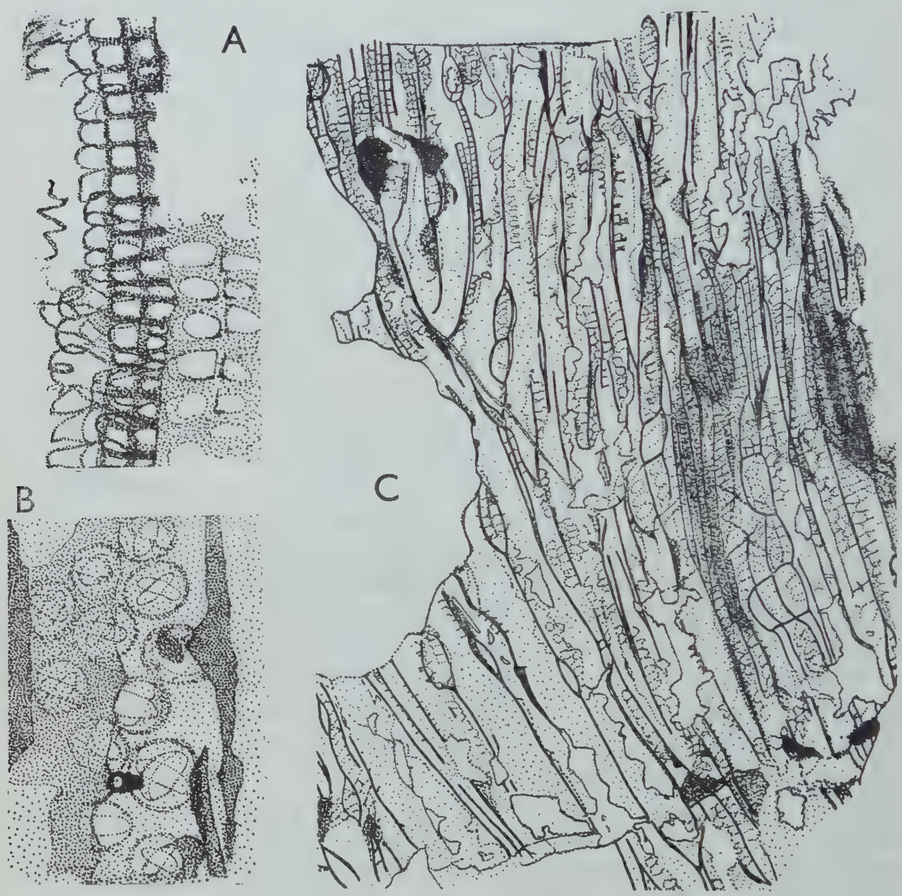
2. *Compact secondary xylem.* The large rectangular areas yield characteristic tracheids in radial view as described by Walton and Wilson and also by Sen (1955). These tracheids, usually about 20 μ to 30 μ but occasionally up to 70 μ broad, show very numerous bordered pits (5–8 μ in diameter) on the whole of their radial walls. The pits are generally multiseriate and are very commonly opposite, often three occurring at the same level (Text-fig. 4 D; Pl. XVIII, Fig. 5). The apertures are usually contracted to a small oblique oval pore and the apertures on the two sides are crossed (Text-fig. 3 B). Rays seem to be rather scarce in this wood, the only ones noticed being one, two and four cells high. The fields where they cross the tracheids have horizontally placed, moderately large, oval pits with very slightly developed borders or none. Walton and Wilson also figured such a ray.

At a few points the compact xylem is seen in tangential section (this has not been seen previously in *Vertebraria*). The tangential walls are almost without any pits (Text-fig. 3 C) but there are a few minute pinhole-like pores which are perhaps true pits. Rarely a few isolated oval pits of the ordinary type may also occur. The tracheids have oblique end walls. The ray cells prove to be rather broad tangentially and the ones seen most clearly proved to be uniseriate and only one to two cells high.

This compact xylem is in contact also with the broad parenchyma rays and here the pitting is quite different (Text-figs. 4 A, B, C; Pl. XVIII, Fig. 4). The pits are mostly large and wide so that the pattern approaches a sort of wide scalariform, but a few circular or polygonal pits also occur. The borders of these pits are only very slightly or not at all developed. At the top and bottom ends of the large rectangular areas masses of tracheids run obliquely or almost horizontally. So far as is known these resemble the vertical tracheids.

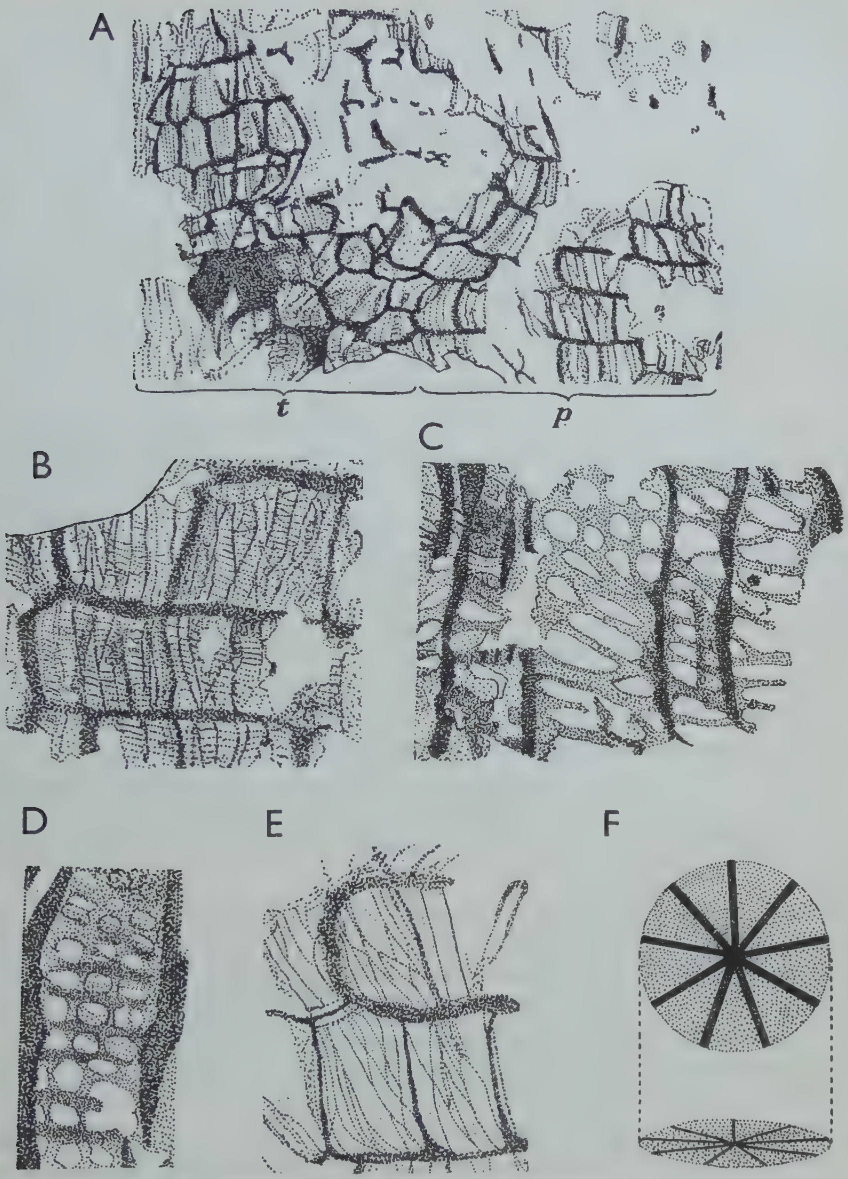
3. *The broad rays.* The existence of these was inferred by Walton and

Wilson but it is now possible to recognize some of their tissue, at least where it is in contact with the compact xylem. The whole of a rectangular area, from top to bottom and from inner edge to periphery, is often occupied by a single sheet of this tissue in perfect radial section and this may even be seen on the surface of the fossil before a peel is prepared (Pl. XVIII, Figs. 7, 8). The cells are very large, usually 133–63 μ (but ranging from 98 μ to 327 μ) high and 82–133 μ radially, rectangular or polygonal in shape, and form well-marked radial files. Their walls are rather thin and show no pits (but as mentioned above the adjacent tracheid walls are characteristically pitted).



TEXT-FIG. 3. *Vertebraria indica*, xylem from pulls. A, protoxylem tracheids and associated early metaxylem, slide 1, × 600. B, radial wall of secondary xylem showing alternate pits with crossed apertures, slide 2, × 1500. C, partly tangential and partly radial view of secondary xylem, showing a few uniseriate rays of various heights, slide 2, × 150.

Walton and Wilson had actually seen a few cells of this tissue, but as they happened to be near the periphery their nature was not recognized with any certainty. From the fact that this tissue flanks the compact xylem for its whole radial extent there is no doubt that it is part of the ray. No definitely



TEXT-FIG. 4. *Vertebraria indica*: A–E, tissue from pulls; F, diagram. A, large parenchyma cells (of main ray) lying over tracheids (*t*) on the left and (?) phloem (*p*) on the right, slide 2, ×50. B, another part of the same ray enlarged, showing parenchyma cells over tracheids, ×150. C, details of pits on tracheids adjacent to a large ray, slide 1, ×600. D, typical secondary xylem pitting, slide 1, ×600. E, large ray cells overlying delicate (?) phloem, slide 2, ×150. F, diagram depicting the mode of compression of a *Vertebraria*.

tangential view of this tissue has been seen and we have no evidence whether other types of cell occurred in it.

4. *Elongated thin-walled cells.* A description of these has already been given by Walton and Wilson who found them running vertically and parallel to the secondary wood and occurring on its outer side. I observed exactly similar cells but my peels were not suitable to show their position in relation to the whole of the *Vertebraria* axis. I noted, however, a large ray which at one point crossed tracheids and at another these thin-walled cells (Text-fig. 4 A). The cells are 15 μ–24 μ in diameter radially. Their length is unknown, but at some points they were seen to have tapering ends. Some of the radial walls show faintly marked pale areas (of about the same size as tracheid pits), but possibly not original features. It seems likely that this tissue may be the phloem.

THE GENERAL CONSTITUTION OF *VERTEBRARIA*

Walton and Wilson held that *Vertebraria* consisted of plates of secondary xylem radiating from a solid central core (there is no suggestion of a pith). Between these plates they believed there were very broad parenchyma rays but of these they found no positive trace, only gaps caused by their disappearance. The tissue of these rays has now, however, been seen, at least in its regions bordering the tracheids. Thin-walled tubular cells, perhaps constituting the phloem, lay on the outer side of the xylem rays.

Walton and Wilson's interpretation receives additional support from the resemblance of *Stigmaria* xylem preserved in the *Gymnostrobus* manner to *Vertebraria*. We see in both the same succession of rectangular areas formed by the alternation of compact xylem (compressed to thin layers of coal) and the less compressed matrix filled spaces corresponding to the original parenchyma of the large rays. The main differences are that *Vertebraria* shows no pith cast and has fewer and wider rays (see Walton and Wilson's restoration (1932, p. 205, Text-fig. 4) of its xylem).

As in *Gymnostrobus* all the features of *Vertebraria* can be explained on the lines of Walton's theory (1936) of compression. Vertical compression of an axis would result in the deformation shown in Text-fig. 4 F and a splitting along the plane of any two almost opposite rays of xylem would result in a *Vertebraria* with two series of rectangles. Such planes, and especially those of nearly opposite rays which are nearest the median horizontal plane, would offer least resistance while splitting and would naturally result in the more common occurrence of *Vertebrarias* showing two series of rectangles. A split nearer the upper or the lower side of the compression, on the other hand, would show three or more series of areas presenting a tangential view of the rays—hence their narrower form as compared to the broad rectangular shape of the areas in axes with two series only.

DISCUSSION

Vertebraria has been discussed by Walton and Wilson and more recently by Sen (1955) who doubted Walton and Wilson's interpretation. I think, however, that Sen had misunderstood Walton and Wilson; the rectangles are the contact areas of secondary ray and tracheidal xylem and are at least as likely to show tracheids as ray cells. All we know of the form of *Vertebraria* is completely consistent with Walton and Wilson's interpretation that it consists of narrow wedges of tracheidal xylem separated by very broad and soft ray tissue. Its rays are so broad that comparison with a food-storing organ is suggested.

The microscopic structure fully confirms their suggestion: all the tissues have now been recognized. Like *Gymnostrobus*, it presents the characters of a xylem core only and, indeed, this may be responsible for the apparent inconsistency between the reports of attached *Glossopteris* leaves on *Vertebraria*, by Zeiller (1896) and Oldham (1897) and on axes which present none of the characters of the genus but which show the external surface and the leaf scars (Etheridge 1894, Pls. 18, 19, and Walton and Wilson, 1932, p. 201, Text-fig. 1).

The rather compact tracheidal xylem of *Vertebraria* is comparable with that of the conifers or such unclassified fossils as *Mesoxylon*, but its pitting is more like that of the pteridosperms than conifers. The tracheids are not as wide as in certain pteridosperms (mostly under 70 μ in diameter) but this is well within the range of tracheids attributed to that class. From the known details of its structure even the pteridophytes are not altogether excluded.

SUMMARY

1. An American specimen (cast) of *Stigmaria* and a *Vertebraria* from Tanganyika have been studied.

2. The American *Stigmaria* has its xylem core preserved in an unusual way, agreeing with the fossil *Gymnostrobus salisburyi* (Lesquereux) Bureau.

3. The features of *Gymnostrobus* are explained as arising from the compression of isolated xylem cores of *Stigmaria*, in terms of Walton's theory of compression.

4. Dry celloidin pulls from the African *Vertebraria* confirm Walton and Wilson's observations showing certain new details of its structure. The tissue of the broader rays in particular has been recognized and this is in accord with Walton and Wilson's inference about their existence.

5. The features of *Vertebraria* are compared with those of *Gymnostrobus* and Walton and Wilson's interpretation is supported on grounds of their similarity as well.

6. The appearance of *Vertebraria* is explained in terms of Walton's theory of compression.

ACKNOWLEDGEMENTS

I owe my warmest thanks to Professor John Walton, Sc.D., F.R.S.E., for providing the material and for his advice during the first part of this investigation which was carried out at Glasgow University, and to Professor T. M. Harris, F.R.S., for his advice and help with the manuscript when I later worked at Reading University.

To Mr. W. N. Edwards, M.A., Keeper of the Geology Department, British Museum (Nat. Hist.) and Mr. F. M. Wonnacott I am thankful for permission to examine the Type specimen and other *Vertebrarias* in their collection and also for facilities to work at the British Museum.

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I also wish to express my thanks to the Committee for Commonwealth University Interchange, British Council and the Government of U.P., India, for financial grants to enable me to visit the U.K.

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DESCRIPTION OF PLATE XVIII

Illustrating D. D. Pant's paper 'On two Palaeozoic axes: *Stigmaria ficoides* in the *Gymnostrobus* condition and *Vertebraria indica*'.

All figures are from untouched photographs.

Stigmaria ficoides (Figs. 1 to 3)

Fig. 1. Cast showing the upper surface with the typical rootlet scars and the dorsal groove. (×½.) Pb 536.

Fig. 2. Xylem core of the same specimen illuminated from the left, showing a pattern of raised ridges on the pith cast and rectangular areas on its two sides. (×1.)

Fig. 3. The same specimen illuminated from above to show the network of coaly lines. (×1.)

Vertebraria indica (Figs. 4 to 8)

Fig. 4. Xylem showing scalariform type of widely oval pits when adjacent to a large ray. (×450.) Slide 1.

Fig. 5. Xylem showing typical multiseriate pits. (×450.) Slide 1.

Fig. 6. Spiral tracheids of protoxylem associated with early metaxylem. (×780.) Slide 1.

Fig. 7. Part of a large rectangular area with carbonized incrustation showing tracheids; illuminated from right. (×30.) Mhukuru coalfield drilling core DH 401/4757.

Fig. 8. Same part, illuminated from top, showing radial files of large parenchyma cells. (×30.)

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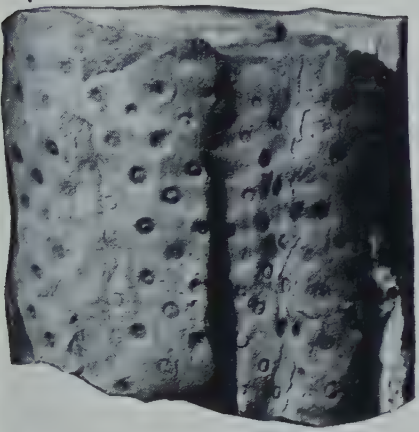

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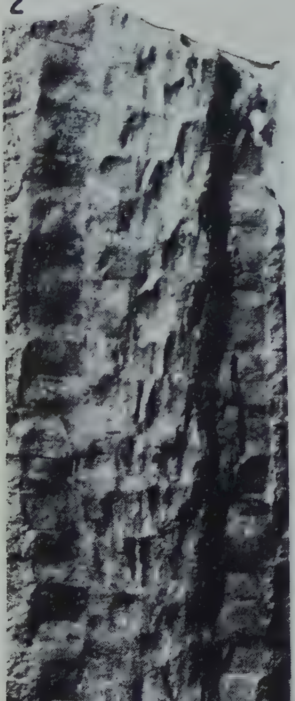

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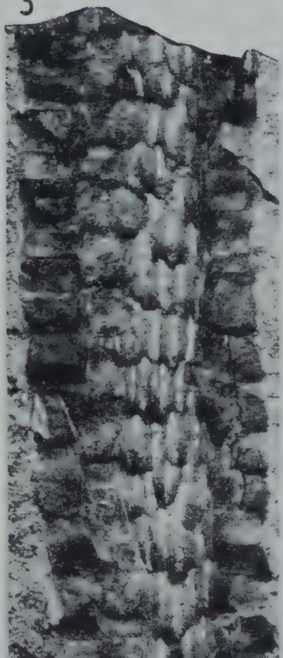

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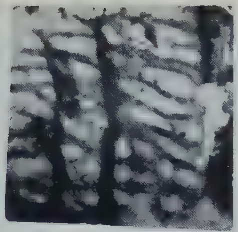

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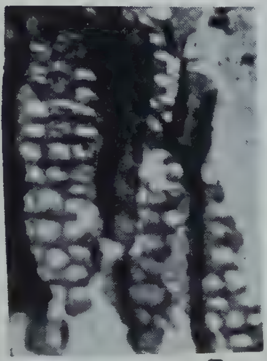

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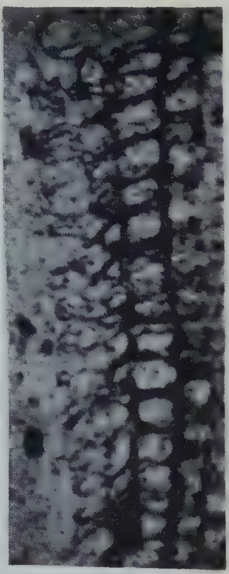

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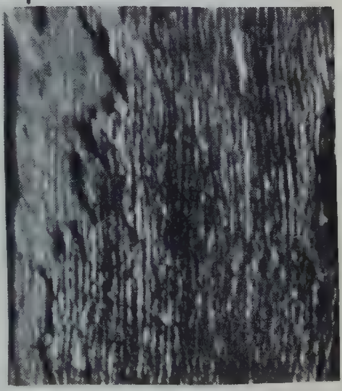

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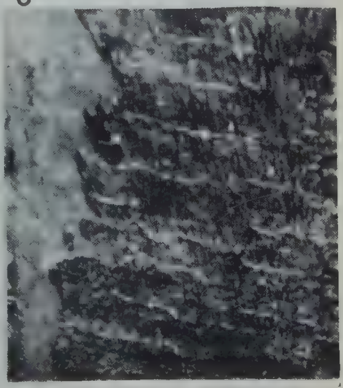


D. D. PANT

Cytological Observations on Three British Species of Laminaria : a Preliminary Report

BY

MARGARET NAYLOR

(*Queen Mary College, London, E. 1*)

With Plates XIX and XX and two Figures in the Text

ABSTRACT

Mitotic divisions in male and female gametophytes and in young sporelings of *Laminaria digitata*, *L. ochroleuca*, and *L. saccharina* from Plymouth follow a normal course and do not show the 'endomitotic multiplication' of chromosomes described by Walker in *L. digitata* from Scotland. Chromosome counts on all three species almost invariably give a value for *n* between 27 and 31, and a definite count of this order will probably emerge.

MATERIAL

THE plants used in this investigation were all collected in the Plymouth region, the *L. digitata* and *L. saccharina* from the rocks at Wembury and the Hoe, and the *L. ochroleuca* from the Millbay Pontoons. Zoospores from portions of mature fronds were germinated and the resulting gametophytes grown on microscope slides in Erdschreiber solution. Continuous illumination gave the most rapid growth and after 12–14 days the gametophytes were mature and liberating their gametes. Plants were fixed *in situ* on the slides either at the stage of oogonium formation or shortly after the liberation of the eggs. Fixation with 3:1; absolute alcohol : glacial acetic acid was followed either by Feulgen's reaction or by acetocarmine staining, using ferric acetate as a mordant. In both cases it was necessary to fix for at least 24 hours, otherwise the plastids tended to stain and obscure the nuclear reactions.

NUCLEAR STRUCTURE

The structure of the nucleus and its behaviour on division is the same in all three species, in gametophytes as well as in sporelings, so one description will suffice.

The resting nucleus. The resting nucleus is granular in appearance, usually with one or two granules larger and more deeply staining than the rest, and possesses a conspicuous nucleolus. With an average diameter of 4·5–5 μ, the nuclei of the female gametophyte and the sporeling are larger than those of the male gametophyte, which have an average diameter of 3·5 μ. The nuclei of the different phases differ in their staining reactions. With Feulgen those of the male gametophyte give an intense coloration, those of the young sporeling a slightly less deep colour, and those of the female only a faint reaction. This difference is seen over a wide range of hydrolysis time and temperature (10–60 minutes at 60° C.; 10–30 minutes at 70° C.; and 5 minutes at 80° C.). A similar difference was noted in *L. digitata* by Walker (1954), who recorded the sporeling as giving the most intense reaction. With acetocarmine the nuclei of the female gametophyte stain less deeply than those of the male and the young sporeling.

Prophase. This is marked by considerable enlargement of the nucleus. In the division prior to oogonium formation it may increase to a diameter of 7·5 μ, and in the young sporelings its diameter may increase until it almost equals that of the cell (see Pl. XIX, D, where resting and late prophase nuclei can be seen).

The chromosomes first appear as fine, convoluted threads with a beaded appearance and condense into progressively shorter and thicker threads until they are short rods about 1 μ long (Pl. XIX, A, B, C, D). Further condensation produces more or less spherical chromosomes evenly distributed throughout the nucleus (Pl. XIX, E); by this stage the nucleolus has disappeared.

In the final prometaphase stage the nuclear membrane breaks down and the chromosomes become fairly widely separated (Pl. XIX, F, G, H; Pl. XX, A and B).

Metaphase. The chromosomes become arranged in a compact metaphase plate (Pl. XIX, J; Pl. XX, C and D). In polar view the individual chromosomes are circular with an average diameter of about 0·5 μ, and in side view they are somewhat elongated (Pl. XX, E). Indications of a spindle are sometimes seen.

Separation of the chromatids; anaphase. The individual chromatids can usually be distinguished in the later stages of prophase, and in the prometaphase stages profile views of the chromosomes frequently show a regular equatorial constriction separating the chromatids (Text-fig. 1, A, B, F). At this stage the structure as a whole is still approximately spherical, but by the time the metaphase plate is organized the pairs of chromatids are elongated in side view and have a dumb-bell shape about 1 μ long by 0·5 μ broad (Pl. XX, E; Text-fig. 1, G and H).

During early anaphase this elongation continues and the isthmus joining the two halves becomes more elongated and tenuous until eventually it ruptures (Pl. XX, F).

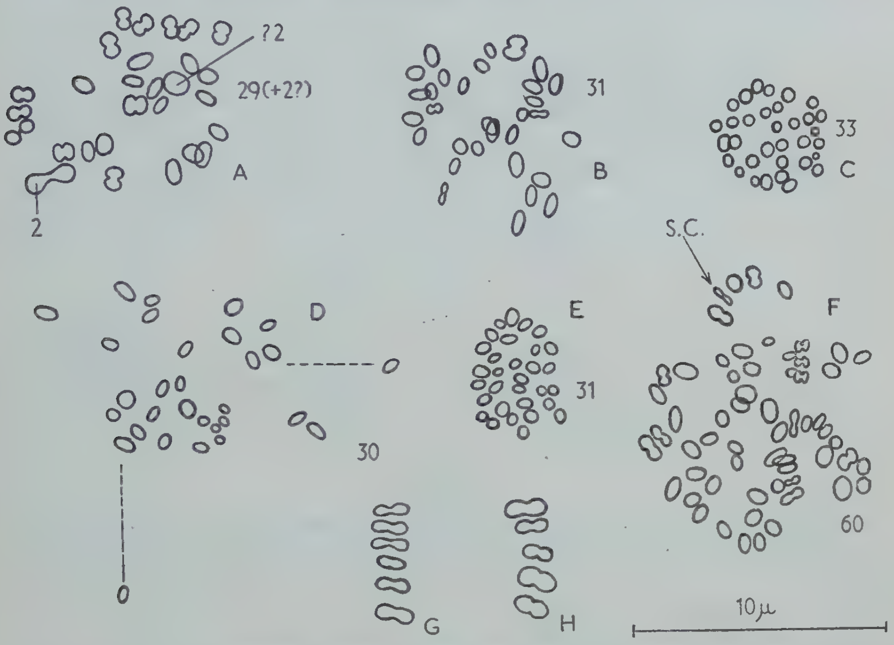
The daughter chromosomes then separate as two uniform plates which pass steadily to the poles, remaining more or less parallel the whole of the way (Pl. XX, G and H). The U- or V-shaped curvature of the chromatids characteristic of most long chromosomes is mechanically impossible in such small chromosomes.

In the early stages of anaphase one or two bridges are present (Pl. XX, F and H). After their rupture the chromosomes concerned lag slightly behind the rest of the plate (Pl. XX, G; Text-fig. 2, C). In polar view the chromosomes appear uniformly circular: one count has been made at this stage (Pl. XX, J; Text-fig. 1, C).

Normal metaphase plates and anaphase separation are seen in all three gametophytes (Pl. XX, H), and there is no indication that 'after the nuclear wall of the male gametophyte has broken down, multiplication of the chromosomes occurs whilst dispersed in the cytoplasm' (Walker, 1954, p. 112).

Telophase. Reconstitution of the daughter nuclei and wall formation follows in the normal fashion in both gametophytes and in the sporelings.

General review. Nothing has been seen in *L. digitata* in support of the view that 'in the single celled stage of the sporophyte the chromosomes undergo endomitotic multiplication to polyploidy whilst dispersed in the cytoplasm; wall formation follows later' (Walker, 1954, p. 115). Pl. XX, A–F show the organization of the metaphase plate and normal anaphase separation of the chromatids in the first division of the sporeling of *L. digitata*. Similar sequences have been seen in the other two species. Telophase and wall formation follow (Text-fig. 2, A, B), and subsequently the two products divide again, either simultaneously or in sequence (Text-fig. 2, C and D), to give a four-celled stage, exactly as described and figured by Kylin (1916) in *L. digitata* and by Sauvageau (1916*b*) in *L. digitata* and *L. saccharina*. These

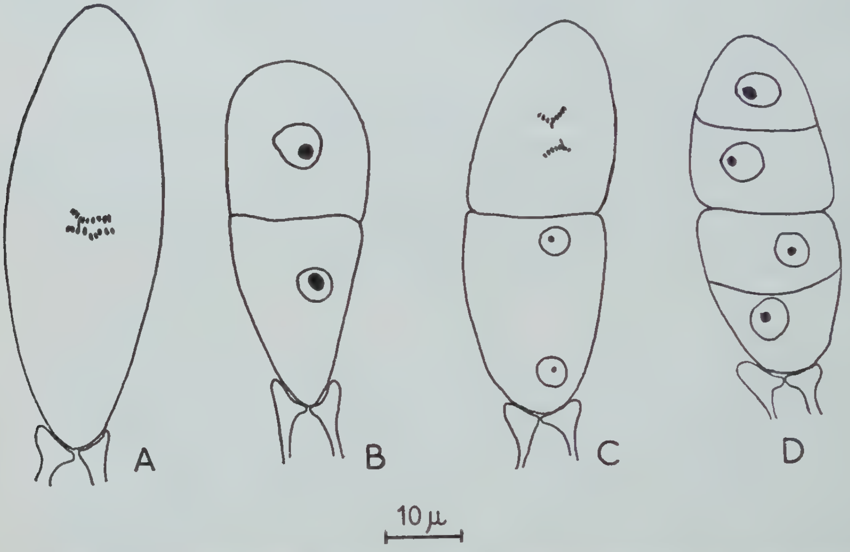


TEXT-FIG. 1. A–F. Tracings of some of the nuclei on which counts have been made, all drawn from photographs enlarged to 10,000 diameters. In each case the count is given at the side. A and B. Prometaphase nuclei of female gametophyte of *L. digitata* (also Pl. XIX, F and G). Some of the chromosomes show separation into chromatids. C. Anaphase plate of female gametophyte of *L. digitata* (Pl. XX, J). D. Extruded egg of *L. ochroleuca* (Pl. XIX, H). E. Metaphase plate of female gametophyte of *L. saccharina* (Pl. XIX, J). F. Prometaphase stage at first division of the sporeling of *L. digitata* (Pl. XX, A). A very small chromosome is indicated by the arrow (S.C.), and a number of chromosomes show separation into chromatids.

G and H. Side views of metaphase plate of *L. digitata* showing the elongation as the chromatids separate.

All to the same scale.

divisions result in the subdivision of the sporeling into a number of cells without any increase in size. The 4, 8, and 16 nucleate sporelings described by Walker as resulting from these endomitotic divisions are probably abnormalities. Similar sporelings were occasionally found in the present series of experiments, but were obviously not following the normal sequence of events, and there was no indication that they had undergone any unusual type of division.



TEXT-FIG. 2. The first division of the sporelings. A. Early anaphase of the first division in *L. digitata* (Pl. XX, F). B. First division completed, *L. saccharina*. C and D. The second division, resulting in a four-celled stage in *L. saccharina*. Comparable stages are seen in *L. ochroleuca*.

All to the same scale, × 1,000 diameters.

THE CHROMOSOME NUMBER

On account of the larger size of the nucleus, counts are most readily made on the cells of the female gametophyte. Although ordinary vegetative cells show all stages in divisions, counts have usually been made during the division prior to oogonium formation when the nucleus undergoes considerable enlargement. This stage is readily recognizable as the cell undergoes a characteristic vertical elongation, often becoming somewhat pyriform in the process. Elongation of a vegetative cell and cutting off of a basal cell prior to oogonium formation, followed by extrusion of the oosphere which is retained on the oogonium wall and subsequently fertilized, has long been recognized (Sauvageau, 1916*a*; Williams, 1921), yet Walker describes this sequence in the following terms: 'After fertilisation of the cell containing the ovum, the diploid complement of chromosomes congregates (Text-fig. 1, G; Pl. 3, C) and passes into an elongation of the oogonium which is then sealed off to form the embryo sporophyte.'

Counts of the diploid number are best made during the first division of the sporeling. The nucleus is at its largest at this stage and becomes progressively smaller during the first few divisions (cf. the sizes of the anaphase plates in Text-fig. 2, A and C) until the normal-sized vegetative nucleus is reached. These are too small for a satisfactory count.

Most of the counts have been made at the prometaphase stage where the chromosomes are fairly widely separated. Most of the metaphase plates are in side view—as is to be expected in a creeping filamentous plant—and where polar views have been found the chromosomes have been too closely crowded to count.

The results of the counts made are listed in the following table:

TABLE I

Chromosome counts made on L. digitata, L. ochroleuca, *and* L. saccharina

	L. digitata	*L. ochroleuca*	*L. saccharina*
Female gametophyte			
prometaphase	30, 27, 31, 29 (+3?), 27 approx., × 29 (+2?), 59	28, 28(+3?), 29, 29	
metaphase			31, 28, 23
anaphase	33		
Extruded eggs			
late prophase	29		
prometaphase	29, 31	30(+1?), 30, 29, 27, 28, 25	27
Sporeling			
First division			
prometaphase	60, 60 approx.		
metaphase	52 approx.		
Later division			
prometaphase	29(+2?)		

The variability of these counts may be partly due to the early separation of the chromatids which is often well advanced by late prophase. This feature, together with the small size of the chromosomes, often makes it difficult to decide whether the unit being counted is a chromatid or a chromosome. Further, some of the chromosomes are appreciably smaller than the rest (Text-fig. 1, F, s.c.), and it is possible that in some nuclei small chromosomes may be hidden by larger ones. Nevertheless, the numbers are all sufficiently close to each other to indicate that a definite count will emerge and that the value of *n* will probably lie between 27 and 31, and that possibly it will be the same for all three species.

A number of counts on extruded eggs give values similar to those on cells of the female gametophytes, so possibly these eggs have not been fertilized. They are usually quite spherical and quite distinct from the normal, elongated young sporelings, and, in the acetocarmine preparations, lack the deeply staining wall characteristic of the fertilized eggs. The 3-celled sporophyte which gave a count of 29(+2?) indicates that sometimes these unfertilized eggs are capable of further development.

The female gametophyte of *L. digitata* which gave a count of 59 could possibly have grown from a spore which had not undergone reduction division.

Text-fig. 1, A–F shows some of the nuclei on which counts were made. All counts were made from tracings of photographs enlarged to ×8,000 diameters, the tracings of different focal planes superimposed and the whole checked by visual observations. Pl. XIX, F, G, H, J; Pl. XX, A and J are photographs of one plane only of the same nuclei.

This estimate of *n* as between 27 and 31 is higher than any value yet recorded either for any species of Laminaria or for any member of the Laminariales (Table II).

TABLE II

Chromosome Counts made on Members of the Laminariales

Species	Worker	Stage investigated	Technique	Value of *n*
Laminaria digitata	Magne, 1953	Sporophyte and unilocular sporangia	Feulgen	13
,, ,,	Walker, 1954	Gametophytes and young sporelings	Feulgen	8
Laminaria hyperborea	Walker, Unpub. (cited Walker, 1952)	—	Feulgen	11
Laminaria japonica	Abe, 1939	Unilocular sporangia	Heidenhain's haematoxylin	22
Laminaria saccharina	Magne, 1953	Sporophyte and unilocular sporangia	Feulgen	13
Chorda filum	Kylin, 1918	Meristoderm cells and unilocular sporangia	Heidenhain's haematoxylin	20
Egregia menziesii	Myers, 1928	Meristoderm cells and unilocular sporangia	Heidenhain's haematoxylin	8
Eisenia arborea	Hollenberg, 1939	Young sporelings	Heidenhain's haematoxylin	15
Macrocystis integrifolia	Walker, 1952	Gametophytes and young sporelings	Feulgen	16
Pterygophora californica	McKay, 1933	Vegetative cells of sporophyte and unilocular sporangia	Heidenhain's haematoxylin	13
Undaria pinnatifida	Inoh and Nishibayashi, 1954	Unilocular sporangia	Heidenhain's haematoxylin	22

The great discrepancy between the counts recorded in the present paper and those given by Magne (1953) and Walker (1954) for the same species may be indicative either of the inadequacy of the methods used for dealing with small chromosomes, or possibly of a genetic difference between the plants from these three localities. Considerably more investigation is needed to determine which is the case.

SUMMARY

1. The chromosomes differentiate as long, slender, convoluted threads which undergo drastic condensation until they are spherical bodies about 0·5 μ in diameter.

2. After a widely spaced prometaphase stage, a compact metaphase plate is organized.

3. Separation into two chromatids begins during prophase by means of an equatorial constriction dividing the chromosome evenly.

4. Normal anaphase separation of two plates of daughter chromosomes and telephase follow.

5. The haploid number of chromosomes is probably between 27 and 31 in all three species.

I wish to thank Dr. M. B. E. Godward for her helpful advice and valuable criticism throughout the course of this investigation. I also wish to acknowledge a grant from the Central Research Fund of the University of London for the collection of material at Plymouth.

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EXPLANATION OF PLATES

PLATE XIX

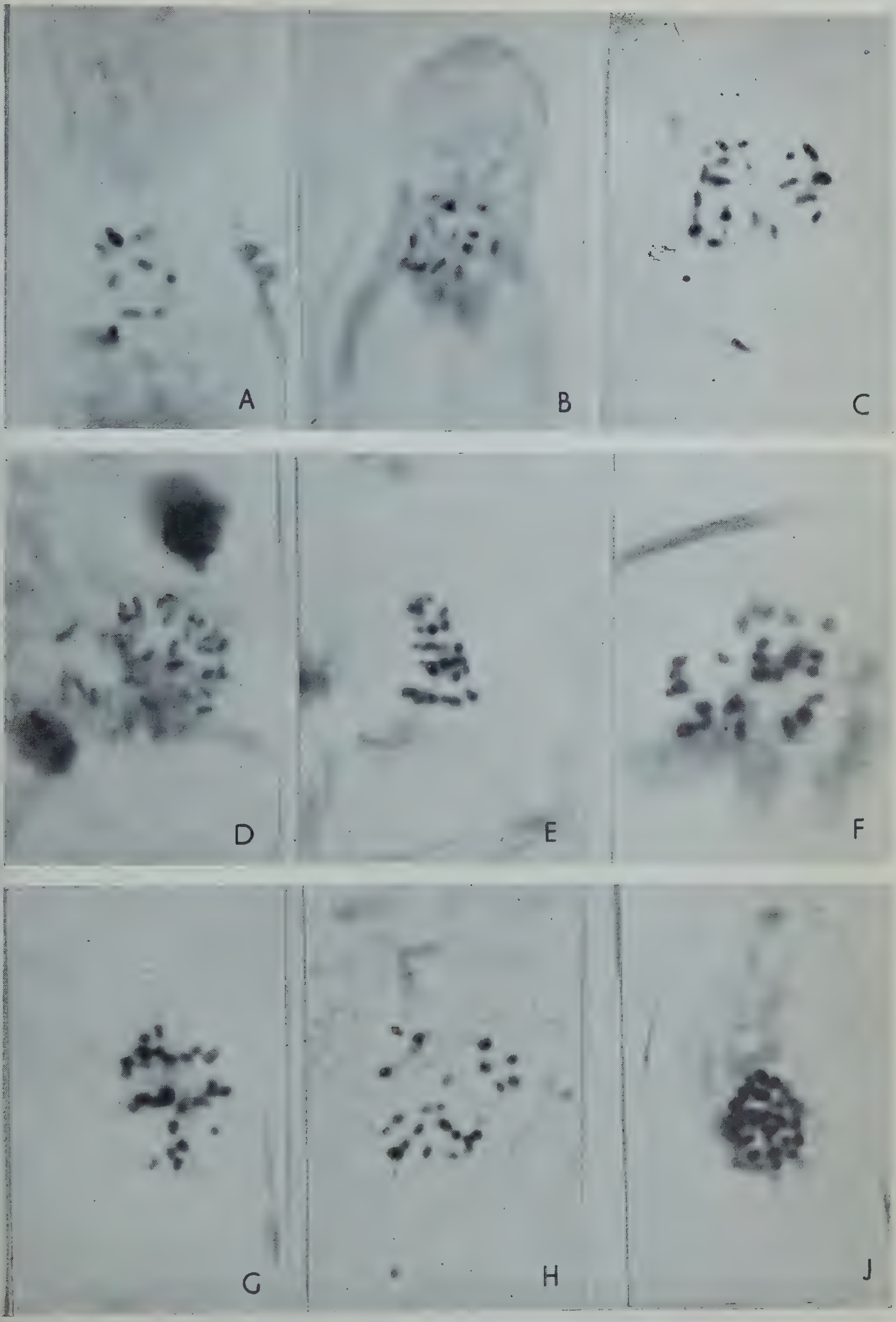
A–D. Late prophase stages showing the chromosomes condensed into short rods. A. Vegetative cell of female gametophyte of *L. digitata*. B. Female gametophyte of *L. ochroleuca*. C. Female gametophyte of *L. saccharina*. D. Cell of young sporeling of *L. ochroleuca* 2 mm. long. E. Later stage in a cell of a young sporeling of *L. ochroleuca* in which the chromosomes are completely condensed into spherical bodies. F–J. Nuclei on which counts have been made, one plane only shown. F, G. Female gametophyte of *L. digitata*. H. extruded egg of *L. ochroleuca*. J. Female gametophyte of *L. saccharina*. Tracings of these nuclei to a higher magnification are shown in Text-fig. 1, A, B, D, E.

A, F, G, J stained with acetocarmine; B, C, D, E, H treated with Feulgen's reagent. All to the same scale, × 3,000.

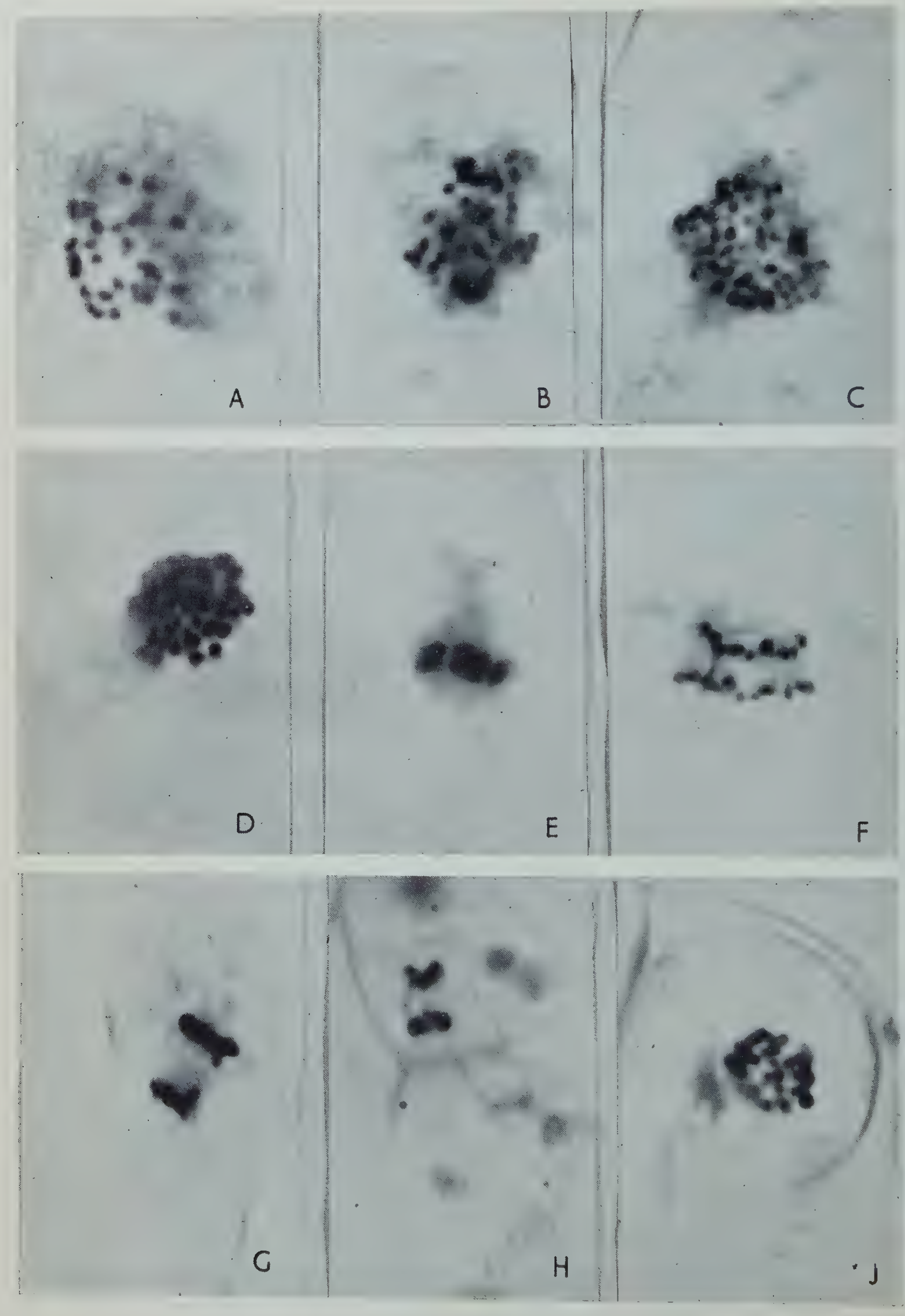
PLATE XX

A–F. Stages in the first division of the nucleus of the young sporeling of *L. digitata*. A, B, C. Three successive stages showing the widely spaced prometaphase chromosomes becoming organized into a compact metaphase plate. A tracing of A is shown in Text-fig. 1, F. D. Metaphase plate, polar view. E. Metaphase plate in side view showing the elongation of the chromosomes and their separation into chromatids. F. An early stage of anaphase showing one bridge. G. Anaphase in a vegetative cell of a female gametophyte of *L. saccharina*: in each plate one chromosome is lagging slightly. H. Anaphase in vegetative cell of male gametophyte of *L. digitata* showing two bridges. J. Polar view of one of the anaphase plates in a developing oogonium of *L. digitata*. This plate gives a count of 33, and a tracing is shown in Text-fig. 1, C.

All stained with acetocarmine and to the same magnification, × 3,000.



M. NAYLOR



M. NAYLOR

The Effects of Synthetic Growth-regulator Treatments on the Levels of Free Endogenous Growth-substances in Plants

BY

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With eight Figures in the Text

ABSTRACT

The possible effects of synthetic auxins and anti-auxins on the metabolism of indole-3-acetic acid (IAA) in plant tissues have not been properly studied in the past. For this reason seedlings of peas, beans, and sunflower have been treated with the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) and two supposed anti-auxins, 2,3,5-tri-iodobenzoic acid (TIBA) and maleic hydrazide (MH), at non-toxic levels sufficient to cause well-marked growth responses. Estimates of the content of alcohol-extractable growth-substances have subsequently been determined, after separation by paper partition chromatography. Although at least six active natural compounds have been indicated in such extracts, only the effects of treatment on IAA levels have been followed in detail.

2,4-D treatment of both leaves and roots has no detectable effect on the levels of free endogenous IAA, and it is thereby concluded that 2,4-D is an auxin in its own right and does not act on growth via a disturbance of IAA metabolism. There are indications that considerable amounts of the absorbed 2,4-D are converted in plant tissues to a neutral detoxication product which is easily decomposed to liberate 2,4-D during chromatographic analysis.

TIBA treatment of pea roots dramatically reduces their free endogenous IAA content, in some cases to 1/10,000 the normal level. The implications of these findings are discussed in terms of the physiological and morphological responses of plants to TIBA treatment.

There are indications that MH may put up slightly the level of free endogenous auxin in pea roots but further confirmatory work is required.

INTRODUCTION

THE rapid, and sometimes bewildering, accumulation of synthetic plant-growth substances in recent years and a fairly exhaustive contemplation of the possible relationships between structure and physiological activity have not been accompanied by a corresponding advance in our knowledge concerning their modes of action. There is indeed a considerable mass of experimental evidence for their competitive interaction somewhere in the mosaic of physiological systems which constitute the machinery of growth. Much of the evidence, however, is conflicting, so that it is virtually impossible to construct a simple unified theory to fit all the facts.

In recent years much emphasis has been laid on a direct action of the many synthetic growth substances at enzymatic centres, which actually control

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growth. 2,4-dichlorophenoxyacetic acid (2,4-D), for example, is such a close homologue of the natural auxin indole-3-acetic acid (IAA) that its action as a growth promoter is usually regarded as taking place on the same enzyme system where it performs a precisely similar role to IAA. Differences in physiological response are usually attributed to indirect modifications of action due to secondary structural properties of the molecule. Similarly, for the range of homologues which antagonize auxin action in various growth phenomena (e.g. indole- and aryloxy-iso-butyric acids, 1-naphthylmethylsulphide-acetic acid (NMSA), 2,3,5-tri-iodobenzoic acid (TIBA), &c.) a competition of active auxin and inactive homologues for the relevant enzyme surface is visualized. Such a competitive action has been postulated for non-homologous growth inhibitors such as maleic hydrazide (MH) and the maleimides (van Overbeek *et al.*, 1955) where there is some evidence for auxin antagonism.

But for many years arguments have been put forward that synthetic growth substances act *indirectly* by altering the level of active natural auxins in the plant. 2,4-D actions were first regarded by Skoog (1947) as arising from competitive release of inactive bound auxin from protein surfaces whereby it becomes available to promote growth. Subsequently the synergistic action of some auxin homologues (e.g. TIBA (Thimann and Bonner, 1948)) has been given a similar explanation. Disturbance of natural auxin metabolism has been suggested as lying at the root of action of auxin antagonists of both the homologous (TIBA) and non-homologous (MH) types (Åberg, 1953; Andreae, 1954). But despite the somewhat optimistic theories erected on all this indirect evidence, no direct attempts were made to determine the effects of these substances on endogenous auxin levels in plants. For this reason the present work was started nearly three years ago. Since its commencement, the results of independent investigations of this kind have been published. Weintraub (1953) has reported that 2,4-D treatment of bean buds lowers the endogenous auxin content. Henderson and Deese (1954) claim that similar responses are shown by etiolated sunflower and pea stems but an increase in auxin content resulted in *Avena* coleoptiles. In both these investigations the *Avena* curvature assay was employed without any attempt to separate 2,4-D from the native auxins in the extract. The assumption was apparently made that, since 2,4-D is almost inactive in this test, it could automatically be assumed that its presence would in no way modify the response to natural auxins. For the time being therefore we must accept these results with reserve until the validity of these assays has been established. Reports by Kulescha (1953) and Pilet (1953) claim that maleic hydrazide treatment causes no change in endogenous auxin levels in *Scorzonera* Crown Gall tissue and lentil roots respectively. Similar uncertainties arise in trying to interpret these findings. On the other hand a recent careful assay of auxins in *Avena* coleoptile using a paper chromatographic technique (Fransson and Ingestad, 1955) has shown that the anti-auxin *p*-chlorophenoxy-*iso*-butyric acid, contrary to expectation, puts up the level of endogenous IAA in treated material.

In the present investigation the effects of three active compounds, each apparently possessing a different type of physiological action, have been studied from this view-point. They are the auxin 2,4-D, the homologous anti-auxin TIBA, and the non-homologous anti-auxin MH. In an attempt to attain precision in the identification and assay of IAA and other auxins in the extract and to avoid the possible sources of error introduced by the presence of the applied compound, the extracts were analysed before assay by a method of paper partition chromatography.

METHODS

(A) *Extraction*

Much preliminary work was carried out on different extraction techniques and the final methods adopted were virtually identical with those described by Bennet-Clark *et al.* (1953) and Kefford (1955 *a*). In essentials this consisted of extracting the macerated plant material in ethanol at 0° C. for 24 hours, evaporation in a partial vacuum to remove the ethanol, acidification with phosphoric acid to pH 3, transference of the auxin to peroxide-free ether in a Kutscher-Steudel extractor, and final concentration of the extract on a water bath to a small volume (about 0·5 ml.). This quantity of solution was then spotted directly on to the starting-line of the chromatogram paper strip by means of a micro-pipette. A series of experiments in which various quantities of IAA were added to tissue homogenates before extracting showed that total losses during extraction, chromatography, and assay were not more than 15 per cent. In some experiments the extracts were first separated into acid and non-acid fractions by shaking with aliquots of 5 per cent. sodium bicarbonate solution. The acid fraction in the bicarbonate was then extracted with ether, acidified to pH 3 with phosphoric acid, and, after concentration, the two portions were run on chromatograms and assayed separately.

(B) *Chromatography and assay*

This method of paper partition chromatography and the associated root-growth assay techniques used in these investigations have already been fully described (Audus and Thresh, 1953). The solvent used was 100 parts iso-propyl alcohol : 10 parts water : 10 parts ammonium hydroxide. Root segment assay was chosen, partly because of its relative simplicity and the reproducibility of its results and partly because of its extra high sensitivity to IAA, it being possible to detect quantities of the order of 10^{-5} μg. It has, however, certain disadvantages, the principal of which is its insensitiveness to such compounds as indole-3-acetonitrile.

Results of each chromatographic analysis were recorded in graphical form and from these graphs the quantities of the various active substances were calculated in terms of IAA equivalents using standard calibration curves. Calculations from the extensive assays performed have shown that the least significant differences to be expected between assay and control means is just under 10 per cent. of the control mean (see also Audus and Thresh, 1953). Any chromatogram strip giving mean root-section growth greater than 110 per cent. or less than 90 per cent. of control has been taken as indicating an active spot. In the majority of assays check chromatograms have been run with pure IAA solutions and sprayed, after drying, with ferric chloride-perchloric acid reagent (Gordon and Weber, 1951) in order to verify the IAA spot in chromatograms of extracts.

Chromatograms have also been run with the three synthetic compounds, alone and in combination with synthetic IAA, to determine their Rf values with the solvent used and to check the possibility of their interference with the running of IAA. The values found fell consistently within the following ranges:

IAA	0·3 to 0·4
2,4-D	0·65 to 0·75
TIBA	0·6 to 0·7
MH	0·15 to 0·25.

The presence of a tenfold excess of any one of the three last compounds in a mixture with IAA had no detectable effect on the Rf of the IAA or on quantitative estimates of concentration using the above-mentioned colour reaction technique.

(C) *Material*

The material used has mostly been of 2–3-week-old seedlings germinated and grown under constant environmental conditions. Sunflower (*Helianthus annuus*), beans (*Vicia faba* and *Phaseolus multiflorus*), and cabbage (*Brassica oleracea* var.) have been grown in soil in wooden flats in a constant temperature room at 25° C. and under a day-length of 14 hours at a constant intensity of 100–120 foot candles produced by fluorescent daylight tubes. This intensity was adequate for normal growth and no etiolation was observed. Peas have been grown under the same conditions in water culture in large tanks supported above the water surface by perforated perspex sheeting. Humidity in the room was not controlled but remained at a low level of about 35 per cent. R.H. Some of the rather older material of bean and cabbage was grown under normal daylight conditions in a greenhouse.

RESULTS

Growth substances in extracts of untreated plants

In the first chromatograms that were run no separations were made of acid and neutral fractions but the whole extract was used in the analysis. The material was of sunflower seedlings and Table I sets out the more important results. This table shows that in sunflower the seed, even after soaking for 24 hours, contains no detectable IAA but that this auxin is rapidly generated during germination. Those samples which included the rather bulky cotyledons contained a much lower IAA content. Separate assays of the cotyledons showed that they contain no detectable auxin.

Three other active spots occurred on the chromatograms. One appeared consistently at Rf 0·10–0·17 and was usually marked by a stimulation of root growth although in the seed extract there was a significant inhibition at this point. This spot corresponds in position to that of accelerator α of Bennet-Clark and Kefford (1953) and has the same root-growth stimulating properties (Kefford, 1955 *b*). Indole-3-pyruvic acid runs at approximately the same place (Stowe and Thimann, 1954). The second consistent spot falls between Rf 0·6 and 0·8 and is usually a strong inhibition. (The stimulation at this point in etiolated plant extracts is interesting but needs further investigation.) The substance is acidic since it is entirely removed with IAA in the acid fraction. It corresponds closely with inhibitor β of Bennet-Clark *et al.* (1953). An inhibitory spot at Rf 0·9 is sometimes found. A typical chromatogram with all five spots is shown in Fig. 1.

Table I

Organ extracted	β—IAA		Rf of other active spots ((+) = stimulation) ((−) = inhibition)			
	Rf	µg./Kg.F.W.				
24-hr. soaked seed	—	0	—	0·20(−)	—	—
10-day shoots	0·40	13·6	—	0·20(+)	0·70(−)	—
14-day shoots and cotyledons	0·32	0·83	—	0·12(+)	0·72(−)	0·92(−)
Ditto	0·32	2·13	0(−)	—	0·80(−)	—
18-day shoots and cotyledons	0·42	1·79	—	0·15(+)	0·72(−)	0·87(−)
Ditto	0·40	2·02	—	0·10(+)	0·80(−)	—
18-day *etiolated* shoots and cotyledons	0·30	11·5	—	0·10(+)	0·62(+)	0·72(−)
18-day shoots	0·40	17·0	—	0·15(+)	0·70(−)	—
Ditto	0·40	16·8	0(−)	0·17(+)	0·67(−)	0·92(−)

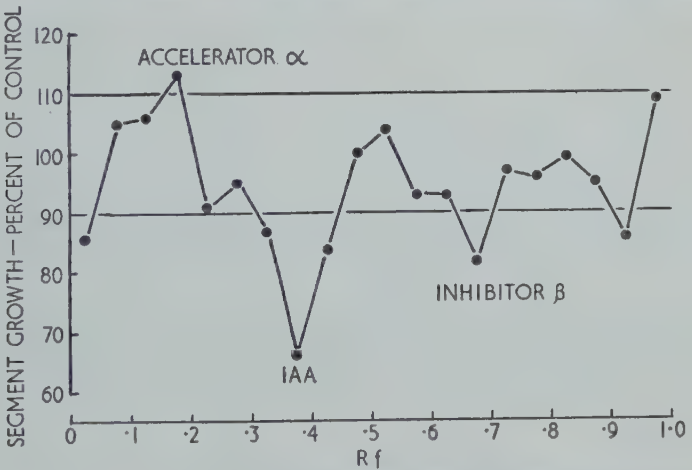

Fig. 1. Graph showing distribution of substances, active in pea-root segment growth, on a paper partition chromatogram of an alcohol extract of sunflower shoots, 18 days from germination.

In pea roots, of which the most extensive analyses have been made, acidic and neutral fractions have been separately analysed. In the acidic fraction, in addition to IAA, inhibitor β (Rf 0·7) has been consistently found together with the second inhibitor at the solvent front (Rf 0·9). In the neutral fraction, on the other hand, with the exception of an occasional inhibition at the solvent front, the only spot was a consistent inhibition at Rf 0·1 and an occasional stimulation just in front at Rf 0·2. It is unlikely that this neutral substance is itself accelerator α of Bennet-Clark and Kefford or indole-3-pyruvic acid, although the spot may be due to either of these compounds produced from a neutral component of the total extract by the action of ammonia in the developing solvent. It is now known that interconversions of this kind can occur (Bentley and Housley, 1956; Nitsch, 1956) and this indicates the need for a re-analysis of such extracts with running solvents not containing ammonia (Nitsch, 1956).

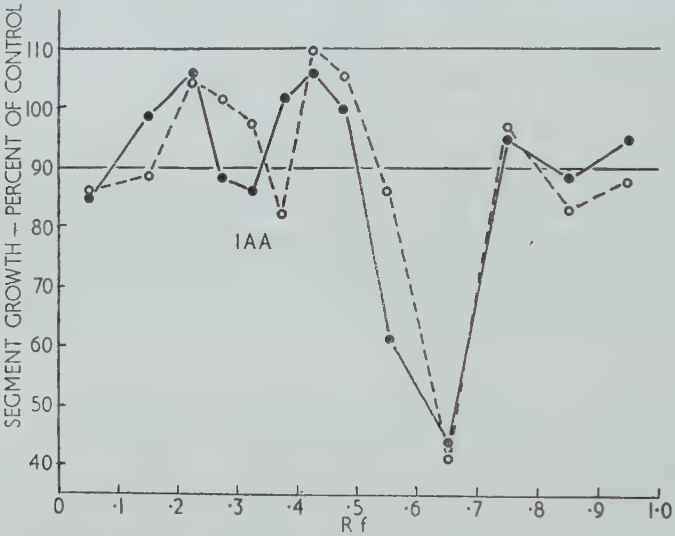

FIG. 2. Graph showing distribution of substances, active in pea-root segment growth, on paper partition chromatograms of two replicate samples from the acid fraction of an alcohol extract of pea roots. Roots from seven-day-old plants.

Analysis of IAA contents of normal seedlings

In order to economize in time and effort, most of the analyses in this investigation have been on that part of the chromatogram containing IAA and estimates of IAA content alone have been made. On a number of occasions final extracts were divided into two equal portions and each half assayed separately. A typical result is shown in Fig. 2. By using the figures from these replicated assays it is possible to get a direct estimate of assay error and to separate it from the variation to be expected between comparable samples of plants. The results are set out in Table II, where A and B represent the replicate assay values.

TABLE II

IAA Content in μg./Kg. F.W.

	SUNFLOWER 14–18-day-old shoots		BROAD BEAN Whole seedlings (17–27 days old)		PEA 7–10-day-old roots		CABBAGE Leaves	
	A	B	A	B	A	B	A	B
	9·5	8·6	0·95	0·87	0·83	0·64	47·4	50·3
	2·5	1·8	0·95	0·93	2·20	1·40	—	—
	16·5	10·75	0·51	0·37	1·70	1·65	—	—
	3·7	2·3	—	—	—	—	—	—
	3·7	2·4	—	—	—	—	—	—
	23·0	18·0	—	—	—	—	—	—
Means	5·96		0·72		1·29		48·7	

These figures, after conversion to logs., have been subjected to an analysis of variance. This appears in Table III.

TABLE III

Analysis of Variance of Log$_{10}$ Conc. IAA in μg./Kg.

Source of variance	Sum of squares	Degrees of freedom	Mean square variance
Species . .	13·9054	3	4·6018
Occasion (sample)	1·9185	9	0·2022
Assay (replication)	0·1098	13	0·0083

This analysis shows that the occasion variance is very much greater than that due to assay error and is therefore highly significant. With these data alone it is not possible to say what proportion of this occasion variance can be attributed to variable extraction losses and other errors due to technique. From the above assay variance it can be calculated that standard deviations attributable to assay errors alone are of the order of 20–25 per cent. of the mean.

The mean IAA contents of the materials calculated from these results appear at the bases of the columns in Table II. Only in pea roots and bean seedlings are IAA levels not significantly different from one another at the 5 per cent. point.

The effects of 2,4-D treatment on IAA content of plants

Various treatment methods have been employed to study 2,4-D effects. In broad bean 0·1 per cent. solutions of the ammonium salt of 2,4-D were brushed over the lowest pair of leaves. Symptoms of epinasty and leaf curl developed after about 24 hours and harvesting for extraction was carried out when these symptoms had developed. In sunflower, seedlings $2\frac{1}{2}$–$3\frac{1}{2}$ weeks old were treated either by painting the cotyledons or the lowest pair of foliage leaves with solutions of the ammonium salt at a concentration of either 0·1 or 0·01 per cent. Both these concentrations produced typical responses in the shoots 24–48 hours later and harvesting for assay was carried out when symptoms were well advanced. Pea seedlings were germinated for

2 days in sterile sand, after which time the radicle only had emerged and was about 4 cm. long. They were then transferred to water culture, one-half of the sample (about 100–150 seedlings) grown thenceforward in distilled water while the second half was grown in solution of the ammonium salt of 2,4-D of concentrations ranging from 0·1 to 0·5 p.p.m. Growth under these conditions was continued for 6–10 days, after which the roots, and in some experiments the shoots separately, were harvested for assay. The effect of

TABLE IV

Material analysed	Concentration of 2,4-D ammonium salt	Point of application	Duration of treatment	IAA conc. in μg./Kg. F.W.	
				Control	Treated
Broad Bean shoots	1,000 p.p.m.	Lower pair leaves	1 day	0·87	0·90
				0·94	1·30
Ditto	Ditto	Ditto	10 days	0·37	0·31
				0·51	0·38
				0·67	0·72
				0·70	
Sunflower shoots 2½ weeks old	1,000 p.p.m.	On cotyledons	1 day	1·6	1·7
	,,	,,	,,	2·5	5·8
	,,	,,	,,	8·1	6·3
	,,	,,	,,	4·2	3·5
	,,	,,	?	7·4	5·4
	,,	On lower pair leaves	9 hrs.	2·4	3·5
				4·4	4·4
Ditto 3½ weeks old	100 p.p.m.	On cotyledons	1 day	1·4	1·4
	,,	,,	,,	2·7	0·7
	,,	On lower pair leaves	,,	3·5	5·3
		Ditto	,,	2·9	5·3
				2·6	3·2
				3·78	
Pea roots (8 days old)	0·5 p.p.m.	Roots	6 days	0·95	0·18
(12 days old)	0·4 ,,	,,	10 ,,	1·08	1·05
(12 ,,)	0·3 ,,	,,	10 ,,	1·03	1·48
(11 ,,)	0·3 ,,	,,	9 ,,	0·81	0·94
(8 ,,)	0·1 ,,	,,	6 ,,	0·27	0·26
				0·83	0·78
				0·80	
Pea shoots:					
(10 days old)	0·3 p.p.m.	Roots	8 days	0·35	0·44
(12 ,,)	0·3 ,,	,,	10 ,,	0·87	0·93
(11 ,,)	0·3 ,,	,,	9 ,,	0·69	0·65
				0·64	0·67
				0·655	

these concentrations of 2,4-D is greatly to reduce the extension of both main and lateral roots (75 per cent. inhibition), but the number of lateral roots is increased by about 60 per cent. The net result is a considerable reduction in the amount of root tissue formed (60–70 per cent. of the total fresh weight). There is no visible effect of these concentrations on shoot growth under these conditions.

In Table IV the results of all these experiments are summarized. Two typical results for sunflower shoots and pea roots respectively are shown in Figs. 3 and 4. It would appear from this table that there are wide occasion-to-occasion variations in IAA content of all material and that this bears no obvious relationship to the age of the material. There are again indications of difference in IAA content in the material of different species but no consistent effect of 2,4-D treatment.

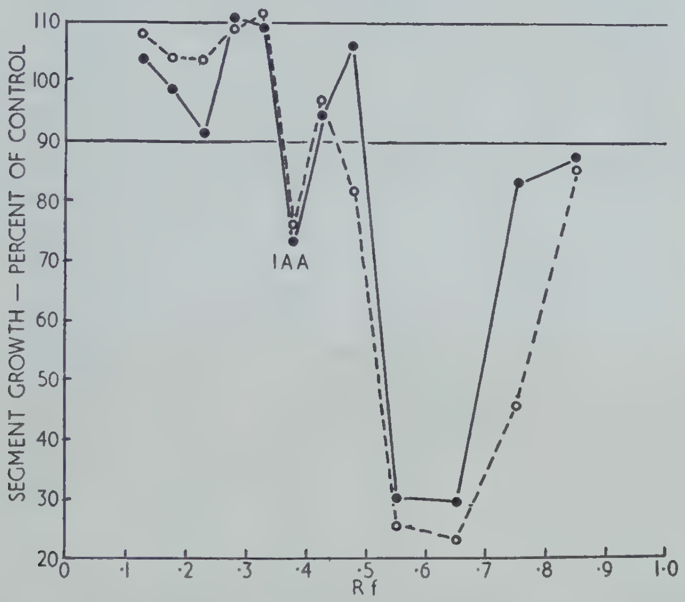

FIG. 3. The effect on the growth-substance content of sunflower shoots of treating the cotyledons of 16-day-old plants with a 0·1 per cent. solution of the ammonium salt of 2,4-D. Graphs showing the distribution of substances, active in pea-root segment growth, on paper partition chromatograms of the acid fraction of alcohol extracts. Period between application and extraction 24 hours. Black points—control shoots (60 g. F.W.); open circles—treated shoots (57·8 g. F.W.).

By lumping all the results from each of the different plant materials and thereby ignoring the difference in treatment and age of plants within each, analyses of variance have been carried out on these data. This was thought to be justified since all the treatments caused typical morphological responses to 2,4-D and if this substance is exerting its action via IAA levels such effects should be apparent in all material and with all treatments.

The results of an analysis of variance carried out after $\log_{10}$ transformation are seen in Table V.

TABLE V

Analysis of Variance of Log_{10} Conc. IAA in μg./Kg. F.W.

Source of variance	Sum of squares	Degrees of freedom	Mean square variance
Material . .	5·318	3	1·773
Occasion . .	2·336	21	0·1112
2,4-D treatment	0·0035	1	0·0035
Material × treatment . .	0·0274	3	0·0091
Residual error .	0·593	15	0·0395

Part of the residual error can be attributed to the assay itself and, as can be seen from Table III, is of the order of 0·008. This leaves an error of the order of 0·03 which can be attributed to variable extraction efficiency, plant sample differences, &c. This gives expected assay variations due to these causes of the order of 35 to 50 per cent. The table shows that there is no significant effect of 2,4-D in any material, since both the treatment variance and the material × treatment interaction are both smaller than the residual variance. The occasion variance, however, is considerably greater than the gross residual variance indicating day-to-day variations in IAA extractable from similar

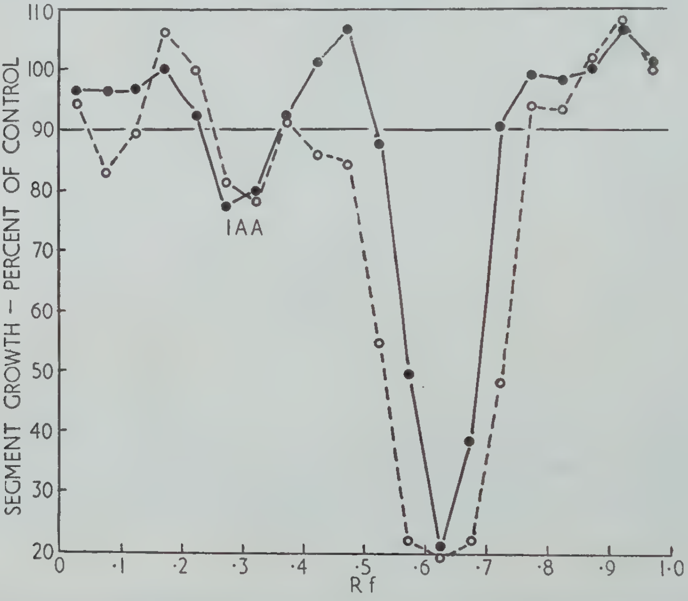

FIG. 4. The effect on the growth-substance content of pea roots of treatment at 10 days from germination with 0·4 p.p.m. of 2,4-D in the culture solution. Graphs showing the distribution of substances, active in pea-root-segment growth, on paper partition chromatograms of the *acid* fraction of alcohol extracts. Period of exposure before extraction 48 hours. Black points—control roots (88 g. F.W.); open circles—treated roots (82·6 g. F.W.).

seedling batches of the same species which cannot be explained by normal assay and extraction errors. It may be that such variations reflect changes in endogenous auxin levels due to uncontrolled variations in the growth conditions employed (e.g. humidity). Alternatively such environmental fluctuations might have caused changes in the ease of extraction of the auxin. But even so the variance due to material is very significantly higher than the total remaining lumped variance. The mean content of IAA in the four species (Table IV) agrees very closely with the results in Table II. The mean levels of IAA in pea roots and shoots and bean shoots do not differ significantly at the 5 per cent. point.

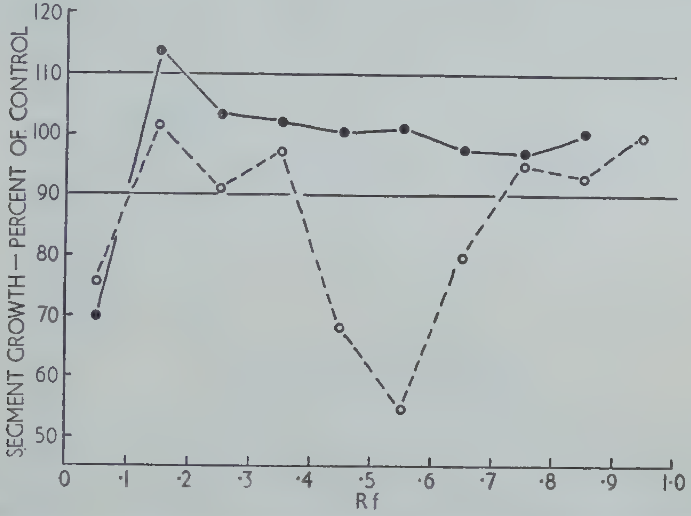

FIG. 5. The effect on the growth-substance content of pea roots of 6 days' growth in a solution containing 0·3 p.p.m. of the ammonium salt of 2,4-D. Treatment started 2 days after germination. Graphs showing the distribution of substances, active in pea-root-segment growth, on paper partition chromatograms of the *neutral* fractions of alcohol extracts. Black points—control roots (92·3 g. F.W.); open circles—treated roots (93·8 g. F.W.).

In many of the pea-root experiments the neutral fractions of the extracts were also run on chromatograms and assayed in the same way. A typical result is shown in Fig. 5. As mentioned earlier, inhibition spots were consistently observed in control extracts at Rf 0·1 and sometimes at Rf. 0·9–1·0. Similar spots were found with treated extracts but in these very marked inhibitory spots were always found (8 experiments) between Rf 0·4 and 0·6. Since these Rf values are near that of 2,4-D it was at first suspected that these spots might have been due to residual 2,4-D left in the neutral fraction as a result of incomplete separation from the acid fraction. This was tested by macerating normal root tissue with 2,4-D solution and then extracting and separating acid and neutral fractions as in the above experiment. On assay no trace of a spot at Rf 0·4–0·6 was found in the neutral fraction.

These spots are therefore due to a *neutral* substance produced in roots by the action of 2,4-D. The most likely explanation is that this substance is a product of the metabolism of 2,4-D by the root tissue.

No consistent changes have been observed in any other spots as a result of 2,4-D treatment. The technique used prevented any observations on the most important of them, i.e. that due to inhibitor β. The 2,4-D spot itself had a very similar Rf value and, owing to the extreme sensitivity of the assay, even the slightest tailing gave rise to a very broad spot of inhibition, thus destroying any chance of detecting the presence of a neighbouring spot.

The effect of 2,3,5-tri-iodobenzoic acid on IAA levels

Experiments on this compound have shown that it is an effective inhibitor of extension growth in attached roots and also in root sections. In pea-root sections 50 per cent. inhibition is caused by a concentrate of about 30 p.p.m. At no concentrations down to and including 10^{-3} p.p.m. has a *stimulation* of root growth been observed (cf. other 'anti-auxins' such as α-(1-naphthyl-methylsulphide)-propionic acid (NMSP) (Åberg, 1950), p-chlorophenoxy-*iso*-butyric acid (PCIB) (Burström, 1950), and 4-chloro-3-nitrobenzoic acid (CNB) (Minarik *et al.*, 1951)). One of its most striking effects on root growth, particularly at slightly lower concentrations (i.e. 10 p.p.m.), is that lateral roots, and to some extent primary roots, become negatively geotropic and grow upwards. This indicates far-reaching effects on the natural hormone system mediating geotropic reactions.

TABLE VI

Duration of treatment (days)	Conc. of TIBA p.p.m.	IAA content µg./Kg. F.W. Control	IAA content µg./Kg. F.W. Treated
6	10	1·3	0·0002*
7	10	1·07	0·0002*
7	10	1·89	0·50
8	10	1·40	0·35
9	10	0·77	0·0001*
8	30	0·51	0 or 0·01†

* Stimulation on chromatogram.
† No response on chromatogram.

Chromatography of stock solutions of TIBA shows it to have an Rf of between 0·6 and 0·7 with the propanol-ammonium-water solvent, thus preventing any possibility of estimating changes in inhibitor β.

In the current investigations 2-day-old pea seedlings were transferred to water-culture tanks and grown for 6 to 9 days on TIBA solutions of 10–30 p.p.m. Roots were then harvested, extracted, and assayed as in the 2,4-D experiments. Details and results of these experiments are shown in Table VI. A typical pair of chromatograms is shown in Fig. 6.

In all these experiments it will be seen that TIBA causes a marked reduction in the cold-extractable auxin content of roots. In three out of the six the level was reduced from the inhibitory to the stimulatory concentration region. Consequently the estimate of IAA content is very rough in these treated samples. This is an inevitable result of using root sections for assay at these very low concentrations. It is possible for example that these stimulations represent the 'tails' of spots at a slightly higher Rf value and having an IAA content giving an assay concentration of about 10^{-9} (i.e. where the assay-curve cuts the control level (see Audus and Thresh, 1953)). Similarly the lack of any observable spot for the 30 p.p.m. treatment may have been due to its being at this concentration level. There is, however, no possible doubt that TIBA has lowered dramatically the alcohol-extractable IAA level in all these experiments. Further extensive experiments with a variety of assay techniques are now indicated to determine the relationships between TIBA dose and the reduction in this IAA level.

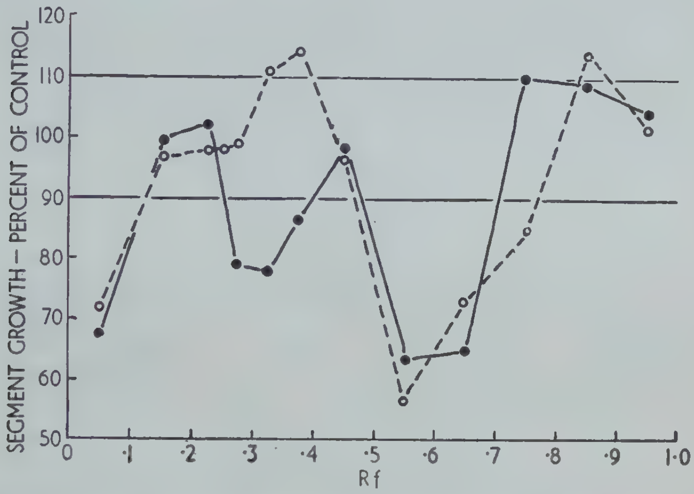

FIG. 6. The effect on the growth-substance content of pea roots of 7 days' growth in a solution containing 10 p.p.m. of TIBA. Treatment started 9 days from germination. Graphs showing the distribution of substances, active in pea-root-segment growth, on paper partition chromatograms of the acid fractions of alcohol extracts. Black points—control roots (91·6 g. F.W.); open circles—treated roots (80·3 g. F.W.).

The effect of Maleic Hydrazide on IAA levels

Maleic hydrazide is also a very strong inhibitor of root growth. When pea seedlings are grown in solutions of its triethanolamine salt both extension growth and the production of laterals are greatly reduced (see Table VII). This contrasts with 2,4-D action which promotes lateral production while inhibiting extension. Very high concentrations are necessary to inhibit extension growth compared with effective concentrations of 2,4-D or even TIBA. The 50 per cent. inhibition of extension growth in sections needs a concentration of 0·15 per cent. On the other hand the growth of attached seedling roots is much more sensitive. A solution of 20 p.p.m. causes a reduction in the number of laterals when applied from 2 days after germination onwards. Similar applications at a slightly later (3-day-old) stage did not cause such a large reduction in lateral numbers. Presumably many laterals had already been initiated before MH was applied. These results indicate very strongly that MH, particularly at these low concentrations, is inhibiting root growth mainly by interfering with the meristematic phase of cell growth. At these concentrations slight inhibition of the growth of shoots was also observed.

In the experiment on IAA levels 2-day-old pea seedlings were grown under the conditions previously described in solutions of the triethanolamine salt of MH at concentrations ranging from 10 to 20 p.p.m. After the treatment period the roots were harvested, extracted, and assayed.

MH has an Rf of between 0·15 and 0·25 with the propanol-ammonio-water solvent used but at the doses employed in these investigations it has no effect on the growth of the assay sections. The level of inhibitor β could thus be determined from the chromatograms as well as that of IAA. Table VII shows the results of all the main experiments carried out. Typical chromatograms are shown in Fig. 7.

TABLE VII

Pea roots

Level of IAA (μg./Kg. F.W.) and Inhibitor β (IAA equiv. μg./Kg. F.W.)

Exp.	Duration of treatment (days)	Conc. MH p.p.m.	IAA		Inhib. β		% Inhibition			Total F.W.
			Cont.	Tr.	Cont.	Tr.	Length of tap roots	No. of laterals	Length of laterals	
5	12	10	0·91	2·0	15·9	10·9	—	—	—	20
6	10	20	0·52	1·65	1·84	2·92	40	55	86	46
7	10	20	0·18	0·20	14·8	too high to estimate	61	57	65	68
8	5	20	0·74	0·37	1·6	5·15	25	50	84	54
9	6	20	0·18	0·97	3·44	2·05	22	55	65	51
Mean	—	—	0·506	1·04	5·69	5·25	37	54	75	48

It will be seen from these few results that there are still large occasion variations particularly marked with inhibitor β. No consistent effect of MH on the level of this compound can be detected. The small differences in the mean are certainly not significant. On the other hand there is a suggestion that MH on the whole has put up the level of free IAA in roots. The mean content for treated roots is just twice that for control roots. Despite the small amount of data a straightforward 't' test shows this difference to be almost significant at the 5 per cent. level whereas the somewhat more precise analysis of variance after $\log_{10}$ conversion shows this MH effect to lie exactly on the 5 per cent. probability point. Further work is obviously called for.

DISCUSSION

The foregoing results demonstrate that, as far as an effect on free ether-extractable IAA is concerned, the three substances investigated exert quite different physiological actions.

2,4-dichlorophenoxyacetic acid

The synthetic auxin, 2,4-D, at concentrations which evoke marked morphological and growth responses in root and shoot, has no consistent effect on free IAA levels. This is in direct opposition to the results of Weintraub (1953) and Henderson and Deese (1954) and the writers venture to suggest that the reasons may be found in the assumptions underlying the assay methods these workers used. Although 2,4-D may be almost inactive in the *Avena* test, nevertheless its presence may affect the sensitivity of that test and so far no data to the contrary have been produced. The data from the present investigation support the theory that 2,4-D is acting *directly* on the growth system and *not indirectly* via a disturbed IAA metabolism. The well-known differences in the responses of plants to high levels of applied IAA and 2,4-D respectively (i.e. toxicity and morphological distortions) are probably not the result of fundamental differences in action but are more likely to be due to a high rate of destruction of IAA in the cell or its immobilization in a bound inactive form. 2,4-D is not inactivated in this way. The experiments indicate, however, that 2,4-D itself undergoes some change in the plant cell and that a fraction is converted to a neutral substance which may itself be an inhibitor or, more probably, is converted into one during the chromatographic analysis or subsequent root-growth assay. This neutral 'detoxication' product could be a simple compound of 2,4-D with a basic cell constituent. Subsequent hydrolysis after fractionation of the extract might have liberated

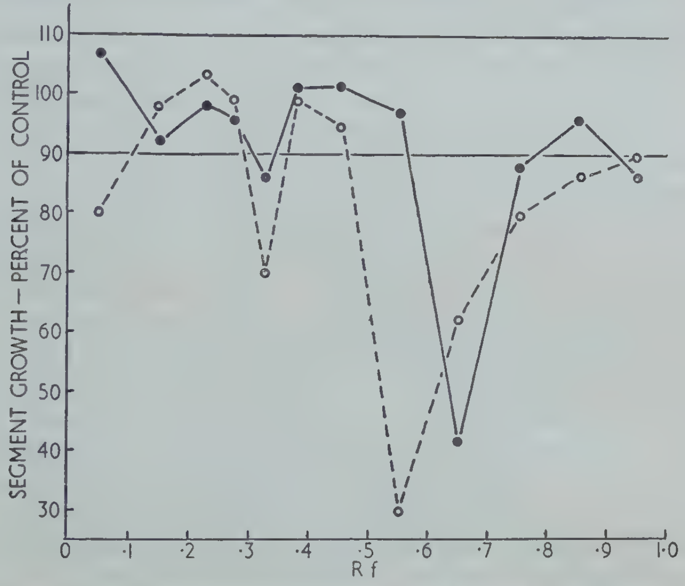

FIG. 7. The effect on the growth-substance content of pea roots of 9 days' growth in a solution containing 20 p.p.m. of MH as the triethanolamine salt. Treatment started 11 days after germination. Graphs showing the distribution of substances, active in pea-root-segment growth, on paper partition chromatograms of the acid fractions of alcohol extracts. Black points—control roots (85·6 g. F.W.); open circles—treated roots (71·3 g. F.W.).

the 2,4-D from this combination since the Rf of the spot it formed was indistinguishable from that of 2,4-D. If this reasoning is correct then the 'detoxication' product can only be formed by enzyme activity in the *living cell*, since it is not found in mixtures of 2,4-D with tissue homogenates.

2,3,5-tri-iodobenzoic acid

The marked reduction in free IAA content caused by TIBA applications at these non-toxic levels would explain a number of the physiological activities of this compound. It would explain its general inhibition of extension growth in internodes (Galston, 1947; Snyder, 1949; Wenck, 1952), its favouring action on abscission (Galston, 1947; Weintraub, Brown, Nickerson, and Taylor, 1952; Whiting and Murray, 1948), and the suppression of apical dominance that has been reported (Snyder, 1949). It would also explain the rather unusual blocking effects observed on IAA transport in sweet-potato petioles (Kuse, 1953) if one can assume that the IAA-destroying effect of TIBA is strictly localized in the treated zone of the petiole. If we can accept the currently favoured postulate that flowering is associated with low auxin levels, then the stimulation of flowering by TIBA (Gorter, 1949 and 1951; Zimmerman and Hitchcock, 1942) would also be explained. If this reduction in IAA level is caused by an enhanced destruction by tissue enzymes then we can explain its antagonism of the action of IAA in the *Avena* test (Galston, 1947; Waard and Florschütz, 1948), and of root-growth inhibition by IAA (Åberg, 1953; Åberg and Jönsson, 1955). Similarly the antagonism by IAA of the effect of TIBA on abscission (Weintraub *et al.*, 1952) can be understood.

However, there are a number of well-authenticated actions of TIBA which cannot be so explained. One is its synergistic action on IAA activity at low concentrations in the *Avena* cylinder test and pea test (Thimann and Bonner, 1948), an action which is supposed to underlie the small auxin activity claimed for this compound (Thimann and Bonner, 1948; Muir and Hansch, 1951). It will not explain the small synergisms of IAA in the inhibition of root growth (Åberg, 1953; Åberg and Jönsson, 1955). The concentration of TIBA used in our experiments (10 p.p.m. $\equiv 2\times 10^{-5}$ M) was of the same order as those which gave growth stimulations and IAA synergisms in coleoptiles and pea epicotyls (Thimann and Bonner, 1948; Muir and Hansch, 1951) and those which give antagonisms of root-growth inhibition by IAA (Åberg, 1953). But in roots the synergistic action on endogenous auxin that has been claimed by Åberg takes place at a much lower concentration. It is possible therefore that in these responses to TIBA we have yet another example of the much higher sensitivity of roots to growth substance, both synergisms and antagonisms setting in at a much lower concentration level in those organs than in coleoptiles and epicotyls. In this investigation only roots subjected to antagonistic concentrations were studied. Further experiments on both shoots and roots with a widely extended range of TIBA concentrations are needed to elucidate this possible double action.

Åberg, in his studies of the action curve of TIBA in flax roots (Åberg,

1953) and pea roots (Åberg and Jönsson, 1955), distinguished three distinct TIBA actions (see Fig. 8). Inhibition in low concentrations (10^{-8} to 10^{-6} M) is postulated as due to synergism of natural auxin (presumed present in supra-optimal concentrations). If this is true, then at this level the depressing effect of TIBA on the IAA content of roots is not being exerted; otherwise a stimulation rather than an inhibition of root growth would be expected. A flattening of the inhibition curve in slightly higher concentrations he supposed to be due to antagonism of the natural auxin and hence a partial relief of its inhibiting action. This corresponds to the concentration used in the current experiments and could be explained by the partial disappearance of natural IAA induced by this TIBA concentration. If this were the only effect of TIBA at this point one would however expect growth inhibition to be reversed and the curve to rise to and even above the control level. Åberg explains the actual behaviour in terms of a rapid onset of 'toxicity' at the slightly higher concentrations, thus presumably preventing the curve from rising.

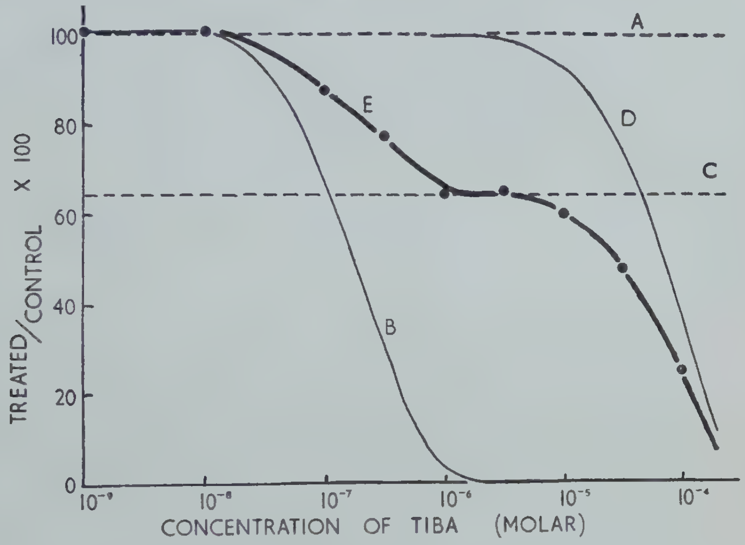

FIG. 8. Theoretical analysis of TIBA action on root growth.
A. Root growth rate with normal levels of endogenous IAA.
B. TIBA effect on endogenous IAA levels.
C. Root growth rate in the absence of endogenous IAA.
D. Curve of direct TIBA inhibition of growth rate.
E. Observed action curve for flax roots (from Åberg, 1953, Table 3, p. 281).
(For further explanation see text.)

This presents a complicated picture of a triple action of TIBA based, to a large extent, on very indirect evidence. Thus its supposed synergistic interaction in low concentrations with natural endogenous IAA is based on the very small synergistic effects obtained with externally applied IAA and 2,4-D. Such small effects could not be expected to give the substantial TIBA inhibitions observed in these low concentrations. Furthermore the explanation presupposes that effective concentrations of endogenous IAA in roots are supra-optimal, a state of affairs by no means established experimentally. Indeed unpublished work in this laboratory suggests strongly that in seedling roots of pea the concentration is sub-optimal. If this is so, then a much simpler explanation of Åberg's stepped curve (E, Fig. 8) can be put forward on a basis of the TIBA effect described in this paper. Thus in the absence of TIBA the root grows at a rate (A, Fig. 8) determined by endogenous IAA which lies either at an optimal or sub-optimal level. As the concentration of TIBA is increased it eventually reaches a point where this endogenous IAA level is lowered progressively (curve B). This results in a corresponding fall in root growth to a lower level C, corresponding to zero concentration of IAA. With further increase in TIBA concentration growth stays at this level until the direct 'toxic' action of TIBA sets in (curve D), when it declines to zero along a typical inhibition curve. The integrated effect of these actions is seen in curve E, which has been fitted to the observed values for flax roots obtained by Åberg (1953, Table III, p. 281).

Only *two* actions of TIBA are necessary therefore to give this stepped curve.

1. A direct inhibiting action in high concentrations resembling that of the true auxins (IAA and 2,4-D) and which Åberg attributes to toxicity.
2. An action, possibly metabolic, greatly reducing the level of free auxin and setting in at a much lower concentration. This action could either be due to a blocking of IAA synthesis or a stimulation of its destruction. The latter seems the least likely since there is one, as yet unconfirmed, report that TIBA inhibits the activity of IAA oxidase (Goldacre, Galston, and Weintraub, 1953). This 'destructive' action may lie at the root of all the TIBA antagonisms of auxin action and phenomena associated with it.

Obviously this explanation too involves many suppositions and the next step in the attack on this problem is a careful study of the TIBA concentration relationships of this IAA-lowering effect, i.e. a verification of curve B in Fig. 8.

Whether a third action, that of *endogenous* IAA synergism, may be operating here and modifying this general picture has still to be established experimentally. TIBA synergisms of *externally applied* auxins, which seem reasonably well established for coleoptile, epicotyl, and root, are probably quite unrelated to the two effects postulated above (1 and 2) and their physiological causes still remain a mystery.

The fact that over this 'plateau' range of TIBA concentration, where IAA levels are lowered, we get such a marked modification of the geotropic response of the root, is a clear indication that IAA plays a fundamental role in such responses.

Maleic hydrazide

The action of maleic hydrazide in plant growth presents a much more confused picture, which is in no way clarified by the results of the current investigations. There is no doubt that it is primarily an inhibitor of cell divisions at concentrations which have little or no effect on extension growth (Deysson and Rollen, 1951; Compton, 1952). These observations are supported by the data presented in this paper where, on the whole, suppression of lateral root production was the most striking feature of maleic hydrazide action at the concentration used. Its well-established action in removing apical dominance has given rise to the suggestion that it is an anti-auxin. Evidence has been obtained of its competitive antagonism of IAA action in standard pea and *Avena* tests (Leopold and Klein, 1952) and of the inhibiting action of IAA in pea roots (Leopold and Klein, 1952) and in flax roots (Åberg, 1953). It has therefore been classified by some workers as a 'true anti-auxin'. This presupposes mutual competitive action at an enzyme surface, but the claim that MH enhances IAA-oxidase activity (Waggoner and Dimond, 1953; Andreae, 1954) would suggest that the antagonism is an indirect one and mediated via IAA metabolic enzymes. On the other hand its ability to cause considerable delays in flowering in a variety of plants, e.g. maize (Josephson, 1951), raspberries (Kennard, Tukey and White, 1951), resembles more the action of an auxin than that of an anti-auxin (cf. TIBA above).

The writers are aware of only two other investigations into the effects of MH on IAA levels. Kulescha (1953) showed that it inhibited growth of crown-gall tissue culture of *Scorzonera* but that the auxin levels in the inhibited tissue were the same as in controls. This suggests that the inhibition is a direct one and not exerted via a metabolic disturbance of auxin levels. Similar claims are made by Pilet (1953) for the action of MH on roots of lentil. In the present investigations on roots, with concentrations sufficient to inhibit both meristematic and extension growth, distinct indications of a raised IAA level were obtained. In view of the marked metabolic disturbance that MH has been shown to evoke in inhibited organs (Greulach, 1953; Phouphas and Goris, 1952; Petersen and Naylor, 1953), presumably the result and not the cause of the inhibition, it is not surprising that auxin levels are also altered. This raising of the IAA level may therefore be purely incidental to the inhibition and have little direct part to play in its cause. Certainly this relatively slight increase observed in the experiments described could not account for the marked growth inhibitions and might be expected to increase rather than decrease lateral root production. It is, however, always possible that the IAA obtained by cold extraction of the tissue bears little relationship to the level of IAA effective in the growth process, but such an argument would seem at the moment to be rather begging the question for the proponents of an anti-auxin action of MH.

There seems little doubt, however, that MH, like TIBA, may be exerting a number of different actions depending on its concentration and the tissue in which it is present. As also for TIBA the final unravelling of these complexities will only come after a careful and systematic survey of these several aspects of their physiological actions, using a wide range of concentrations and an appropriate and representative selection of plants and plant organs.

Acknowledgement

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The *In Vitro* Action of Griseofulvin against Pathogenic Fungi of Plants

BY

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With Plate XXI

ABSTRACT

The action of griseofulvin *in vitro* against forty economically important fungal pathogens of plants has been examined. The pathogens were assigned to one of four groups, according to the severity of the morphological symptoms observed. It was concluded that griseofulvin has considerable potential value for the protection of plants against a wide range of fungal diseases.

INTRODUCTION

IT is now sixteen years since the antibiotic, griseofulvin, was first isolated and nine since the discovery of its interesting anti-fungal properties, but it still remains to be evaluated as a plant protectant in agriculture. Discovered and isolated by Oxford, Raistrick and Simonart (1939) as a metabolic product of *Penicillium griseofulvum* Dierckx in 1939, it was also isolated by Brian, Curtis, and Hemming (1946) as the antibiotic 'curling factor' from *P. janczewskii* in 1946. Grove and McGowan (1947) subsequently showed that 'curling factor' and griseofulvin were identical. Its chemical structure was established by Grove, Ismay, Macmillan, Mulholland, and Rogers (1951) and its biological activity was studied by Brian (1949). He concluded that it profoundly affected the morphogenesis of Basidiomycetes, Ascomycetes, Fungi Imperfecti, and Zygomycetes (Mucorales), 'causing stunting, excessive branching, abnormal swelling and distortion of hyphae and production of spirally twisted hyphae' at concentration of 0·1–10 μg./ml., but had no effect on the Oomycetes (Saprolegniales and Peronosporales) and certain yeasts. Brian therefore suggested that griseofulvin was only effective against organisms with chitinous cell walls.

The potentialities of griseofulvin for controlling pathogenic fungi in plants was first demonstrated by Brian, Wright, Stubbs, and Way (1951): they concluded that it possessed undoubted activity as a systemic 'fungicide' against *Alternaria solani* on tomato and *Botrytis cinerea* on lettuce. No reports, however, have yet appeared about its field performance.

Griseofulvin can now be produced by submerged fermentation, in quantities adequate for field trial: the procedure is the subject of Patent applications. We have examined the action of griseofulvin *in vitro* against a range of economically important fungal pathogens of plants, with the object of obtaining some indications as to which plant diseases might be expected to be brought under control by treatment with it in the field.

[Annals of Botany, N.S. Vol. XX, No. 79, July 1956.]

MATERIALS AND METHODS

Thin, permeable cellophane discs (2×2×0·04 cm.) were soaked in griseofulvin solutions of the required concentration, blotted to remove free solution from the surfaces and then transferred on to pads of three 7 cm. filter papers saturated with 2·2 ml. of the same solution of antibiotic and contained in a Petri-dish. The upper surfaces of the cellophane discs were seeded with either spores or mycelium of the test fungus and were then incubated at room temperature until new hyphae had developed from the inoculum. The cellophane discs were mounted (mycelium downwards) on a microscope slide, a drop of water was applied to the cellophane to remove air bubbles, and the whole preparation was sealed with a coverslip and Canada balsam so as to give a semi-permanent preparation.

The effects produced by 0, 0·3, 0·6, 1·25, 5, 10, 20, and 40 μg. griseofulvin per ml. were viewed under the microscope and then recorded by photomicrography. Four distinct types of morphological change were observed and the minimum griseofulvin concentration inducing any of these responses was noted for each pathogen.

Fungi failing to show a morphological response to griseofulvin were tested for the presence of chitin by means of the van Wisselingh chitosan test, as described by Nabel (1939). A positive result for chitin was obtained on a few griseofulvin-insensitive species: these species were then subjected to a further test for griseofulvin activity by determining their growth curves (dry weight) in submerged culture, in the presence of the antibiotic at a concentration of 40μg./ml. or in its absence.

RESULTS

The responses of the fungal pathogens to griseofulvin showed wide variation but could, in general, be grouped into the following four categories:

1. Curled hyphae at very low griseofulvin concentration, and then progressively excessive branching, stunting, and gross malformation of hyphae with increased concentration of antibiotic.
2. Curled hyphae at low griseofulvin concentration progressing to excessive branching and growth inhibition with increase in antibiotic concentration. Absence of gross hyphal distortion.
3. Curled hyphae but no other morphological change.
4. Absence of any morphological change up to a griseofulvin concentration of 40 μg./ml.

Representative fungi from each of the three groups showing a response to griseofulvin are illustrated in Plate XXI; the complete results are tabulated in Table I.

Among the fungi that showed no morphological response to griseofulvin (group 4), *Pythium debaryanum* and *P. ultimum* proved to be negative in the van Wisselingh chitosan test, whereas *Elsinoe ampelina*, *Exobasidium vexans*, and *Ustilago maydis* gave a positive reaction. Despite the presence of apparent chitin in *Exobasidium vexans* and *Ustilago maydis*, griseofulvin had no effect on their dry-weight growth curves in submerged culture, thus confirming the observations on surface growth. The effect of griseofulvin on the growth curve of *Elsinoe ampelina* was not examined, because of the unsatisfactory growth of this organism in submerged culture.

DISCUSSION

Present knowledge does not indicate the significance of various morphological effects in relation to protection of the host plant: though it might be expected that pathological species undergoing the gross malformation seen in representatives of group I, or the excessive branching and growth inhibition found in group II, would fail to proliferate in the griseofulvin-treated plant, it is much less obvious what degree of protection might result with fungi of group III, which only respond to griseofulvin by 'curling'. This point merits further investigation, since it is possible that the 'curling' response alone may militate against entry to the host plant and may have various degrees of importance, according to the mode of penetration.

The absence of any response to griseofulvin in *Elsinoe ampelina, Exobasidium vexans*, and *Ustilago maydis* is of particular interest in view of the fact that these three species appear to have chitinous cell-walls. Clearly, therefore, Brian's (1949) postulate, that griseofulvin is specific against organisms with cell-walls composed of chitin, needs to be modified. It is noteworthy that the three species not conforming to this hypothesis, as also the two anomalous yeasts (*Torulopsis utilis*, *Saccharomyces cerevisiae*) mentioned by Brian (1949), all exhibit a fragmenting type of growth in which the filamentous form is either absent or transient. It may be that in such organisms the walls of the individual cells mature more rapidly than the wall in the apical region of filamentous fungi and that griseofulvin can only exert its effect on immature chitinous cell-walls. Such an hypothesis would accord with our experience that in filamentous forms, griseofulvin is more active on young than on old hyphae.

Although *in vitro* results do not necessarily indicate the probable efficacy of griseofulvin in the field, it is clear that this systemic antibiotic has considerable potential value in the protection of plants against a wide range of fungal diseases. The many novel properties associated with griseofulvin, however, suggest that much field experimentation will be needed before its utility can be finally assessed.

SUMMARY

The action of griseofulvin *in vitro* against forty economically important fungal pathogens of plants has been examined.

The pathogens showed considerable differences in response to griseofulvin and were assigned to one of four groups, according to the severity of the morphological symptoms observed. Only five fungi were unaffected by

TABLE I

Action of griseofulvin on pathogenic fungi 'in vitro'

	Type of inoculum Mycelium (M) or Spores (S)	Minimum concentrations of griseofulvin to produce morphological effects described (μg./ml.)				
		Curling	Excessive branching	Stunting	Gross hyphal distortion	Complete inhibition
Group I—Curled hyphae at very low griseofulvin concentration followed progressively by excessive branching, stunting and gross malformation of hyphae with increased concentration of antibiotic.						
Basidiomycetes (Hymenomycetales)						
Corticium solani (Bottom rot of lettuce, &c.)	M	0·3	1·25	2·5	5	20
Ascomycetes						
(i) Erysiphales						
Diplocarpon rosae (Black spot of rose)	M	2·5	5·0	10	20	..
(ii) Sphaeriales						
Glomerella cingulata (Bitter rot of apple)	S	0·3	0·6	2·5	10	..
Venturia inaequalis (Apple scab)	S	0·6	..	5	10	..
**Venturia pirina* (Pear scab)	M	0·3	1·25	2·5	20	..
(iii) Pezizales						
Sclerotinia homoeocarpa (Dollar spot of turf)	M	0·3	1·25	2·5	10	..
,, *camelliae* (Petal blight of camellia)	M	0·3	0·6	1·25	2·5	..
Deuteromycetes						
(i) Hyphomycetales						
Alternaria solani (Early blight of tomatoes)	S	0·3	1·25	2·5	5·0	..
Botrytis allii (Onion rot)	S	0·3	..	1·25	2·5	10
,, *cinerea* (Grey mould of lettuce, &c.)	S	0·3	1·25	2·5	5·0	20
,, *fabae* (Chocolate spot of broad beans)	S	0·6	1·25	1·25	2·5	20
,, *tulipae* (Tulip fire)	M	0·3	1·25	1·25	2·5	20
**Clasterosporium carpophilum* (Shot hole of peach)	S	0·6	2·5	5	10	..
Helminthosporium avenae (Leaf stripe of oats)	M	0·3	2·5	5	10	..
Verticillium dahliae (Tomato wilt)	S	0·3	0·6	20	40	..
Sporocybe azaleae (Bud blast of rhododendron)	M	0·3	0·3	0·6	1·25	20
(ii) Sphaeropsidales						
Deuterophoma tracheiphila (Root rot of citrus)	M	0·3	0·6	0·6	10	..
**Diplodia natalensis* (Die-back of citrus)	M	0·3	0·6	2·5	40	..
Phoma betae (Black leg of sugar-beet)	M	0·3	2·5	5	10	..
(iii) Melanconiales						
Colletotrichum lagenarium (Anthracnose of cucumber)	M	1·25	2·5	10	20	..

966.79

Group II—Curled hyphae progressing to excessive branching and growth inhibition with increase in griseofulvin concentration. Absence of gross hyphal distortion.						
Basidiomycetes (Hymenomycetales)						
Armillaria mellea (Root rot of conifers)	M	0·3	2·5	5	..	..
Stereum purpureum (Silver leaf of plum)	M	0·6	2·5	20	..	..
Ascomycetes						
(i) Hypocreales						
Nectria galligena (Apple canker)	M	1·25	5·0	20	..	..
(ii) Sphaeriales						
**Didymella lycopersici* (Stem rot of tomato)	S	0·3	20	40	..	..
Deuteromycetes						
(i) Hyphomycetales						
Cercospora melonis (Blotch of cucumber)	M	0·3	2·5	20	..	..
,, *musae* (Banana leaf spot)	M	0·6	5	10	..	..
Fusarium caeruleum (Potato dry rot)	S	0·6	2·5	5	..	..
,, *culmorum* (Die-back of carnations)	S	0·6	2·5	5	..	..
,, *nivale* (Snow mould of turf)	S	0·6	2·5	10	..	..
,, *oxysporum var. cubense* (Panama disease of banana)	M	0·3	1·25	10	..	..
Verticillium albo-atrum (Tomato wilt)	S	1·25	2·5	5	..	..
,, *cinerescens* (Carnation wilt)	S	0·3	2·5	5	..	..
(ii) Sphaeropsidales						
Septoria apii-graveolentis (Leaf spot of celery)	M	2·5	20	40	..	..
Group III—Curled hyphae but no other morphological change.						
Basidiomycetes (Hymenomycetales)						
Ganoderma applanatum (Wound parasite in various trees)	M	20	..	..	..	..
Deuteromycetes (Hyphomycetales)						
Cladosporium fulvum (Tomato leaf mould)	S	0·6	..	..	..	..
Group IV—Absence of any morphological change up to 40 μg. griseofulvin per ml.						
Basidiomycetes						
(i) Ustilaginales						
Ustilago maydis (Maize smut)	M	..	..	..	..	..
(ii) Hymenomycetales						
Exobasidium vexans (Blister blight of tea)	M	..	..	..	..	..
Ascomycetes (Plectascales)						
Elsinoe ampelina (Anthracnose of vine)	M	..	..	..	..	..
Phycomycetes (Peronosporales)						
Pythium debaryanum (Damping-off diseases)	M	..	..	..	..	..
,, *ultimum* (Damping-off diseases)	M	..	..	..	..	..

* Morphological changes observed rather less severe than in typical members of the group.

griseofulvin, but these included three species that appeared to have chitinous cell walls. This led to the suggestion that griseofulvin can only exert its effect on immature chitinous cell walls.

It was concluded that griseofulvin has considerable potential value for the protection of plants against a wide range of fungal diseases.

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EXPLANATION OF PLATE XXI

Illustrating the paper on the action of griseofulvin by Eunice J. Napier, Dorothy I. Turner, and A. Rhodes

Characteristic morphological changes produced in representative fungi from groups I, II, and III on spore germination in presence of increasing griseofulvin concentrations. All photomicrographs ×400.

Figs. 1–4. *Botrytis cinerea* (Grey mould of lettuce).
- Fig. 1. Control. No griseofulvin.
- Fig. 2. 0·6 μg./ml. griseofulvin. Curled hyphae.
- Fig. 3. 2·5 μg./ml. griseofulvin. Growth inhibition, curling and excessive branching.
- Fig. 4. 5 μg./ml. griseofulvin. Gross hyphal distortion.

Figs. 5–7. *Fusarium caeruleum* (Dry rot of potato).
- Fig. 5. Control. No griseofulvin.
- Fig. 6. 0·6 μg./ml. griseofulvin. Curled hyphae.
- Fig. 7. 40 μg./ml. griseofulvin. Growth inhibition, curling and excessive branching.

Figs. 8–9. *Cladosporium fulvum* (Tomato leaf mould).
- Fig. 8. Control. No griseofulvin.
- Fig. 9. 0·6 μg./ml. griseofulvin. Curled hyphae.

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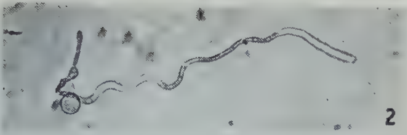
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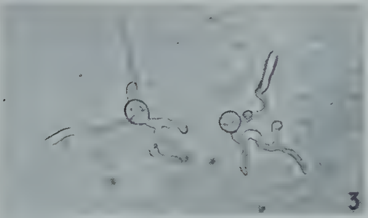
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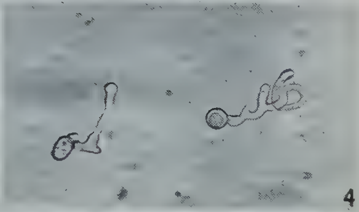
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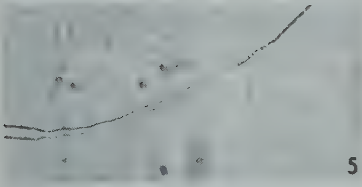
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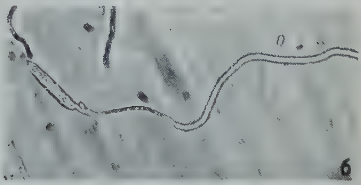
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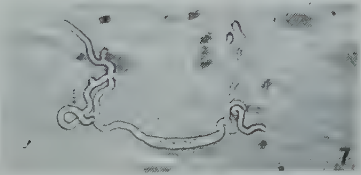
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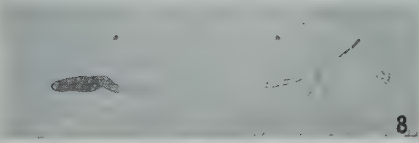
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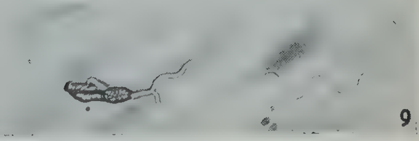
9

E. J. NAPIER, D. I. TURNER, *and* A. RHODES

The Influence of Light and Temperature on Fruiting of *Coprinus lagopus* Fr. in Pure Culture

BY

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With Plate XXII and one Figure in the Text

Abstract

Some effects of light and temperature on fruiting of *Coprinus lagopus* in pure culture are described. Fruiting, which in darkness did not commence until about the 15th day, was accelerated by continuous light or by brief exposures to light between the 7th and 13th days of incubation. Very small exposures sufficed, provided they were of a wavelength no longer than that of green light, the response being restricted to the area of mycelium actually exposed. In both light and dark a temperature near 25° C. was optimal for growth and fruiting.

Introduction

It was noted in an earlier contribution (Madelin, 1956) that fruiting of an isolate of *Coprinus lagopus* was delayed by darkness. This was in contrast to Voderberg's (1949) report that initiation of fruiting in this species is virtually independent of light, if anything being slightly retarded by it. Light has, however, been found necessary for sporophore initiation in certain other species of *Coprinus*, as for example in *C. nycthemerus* (Brefeld, 1889). This effect of light must be distinguished from its formative effect on the development of the individual fruit-body, of which the literature contains many accounts. Thus Borriss (1934*b*) and Voderberg (1950) record that light influences the development of the fruit-bodies of *C. lagopus* subsequent to their initiation, and they and others describe how in darkness the fruit-bodies develop long, slender stipes and generally only rudimentary pilei. Both as a tropic and formative stimulus acting on *C. lagopus*, only light of the blue range of the visible spectrum is effective (Borriss, 1934*b*). Brefeld (1889) noted a similar sensitivity to the colour of light in *C. plicatilis*.

Voderberg (1949) reports that the optimal temperature for growth and fruiting of *C. lagopus* is between 20 and 25° C. and that no fruit-bodies develop above 30 and below 10° C., although mycelial growth continues weakly. Similar results were obtained in both the light and the dark.

In this communication are described some aspects of the influence of light and temperature on chiefly the fruiting of cultures of *C. lagopus*.

Material and Methods

The isolate of *C. lagopus* and the methods of growing it and of determining yields of vegetative mycelium and sporophore tissue were those used in earlier nutritional studies (Madelin, 1956).

[Annals of Botany, N.S. Vol. XX, No. 79, July 1956.]

In one experiment described below, the fungus was cultured on a compact layer of horse dung, about 5 mm. deep, in a Petri-dish, autoclaved for 20 minutes at 15 lb./sq. in. pressure. In all other cases the substrate employed was an agar medium of the following composition:

Glucose	1%
dl-α-Alanine	0·1%
K_2HPO_4	0·2%
$MgSO_4 \cdot 7H_2O$	0·02%
Thiamin hydrochloride	500 μg. per 1,000 ml. of medium
Agar	2%

—in distilled water.

The phosphate was sterilized separately and added aseptically to the rest of the medium after autoclaving. Ten-ounce medicine bottles were used as culture vessels, each containing 20 ml. of the above agar medium in the form of a thin layer on the broad inner face of the bottle.

Media were inoculated with plugs of mycelium-bearing agar, 1 mm. in diameter, taken from near the margins of plate cultures. The inoculum was placed centrally upon the medium except in those cases where growth was to be followed gravimetrically by means of a sampling technique. In such cases it was placed half-way along one side of the medium contained in a medicine bottle, thereby enabling the subsequent semi-circular colony to grow for a much longer period before meeting any physical obstruction.

Cultures were incubated in a constant-temperature room at 25° C. and illuminated at an intensity of about 8 foot candles at the culture surface with light provided by an incandescent tungsten filament bulb. Unless stated to the contrary in the text, cultures to be maintained in total darkness were transferred immediately after inoculation to light-proof cabinets in the constant-temperature room. To ensure adequate aeration, these cabinets were opened briefly at frequent intervals after completely darkening the room. There was no evidence to suggest that these brief periods of darkness had any effect on other cultures in the same room which were ostensibly subject to continuous illumination.

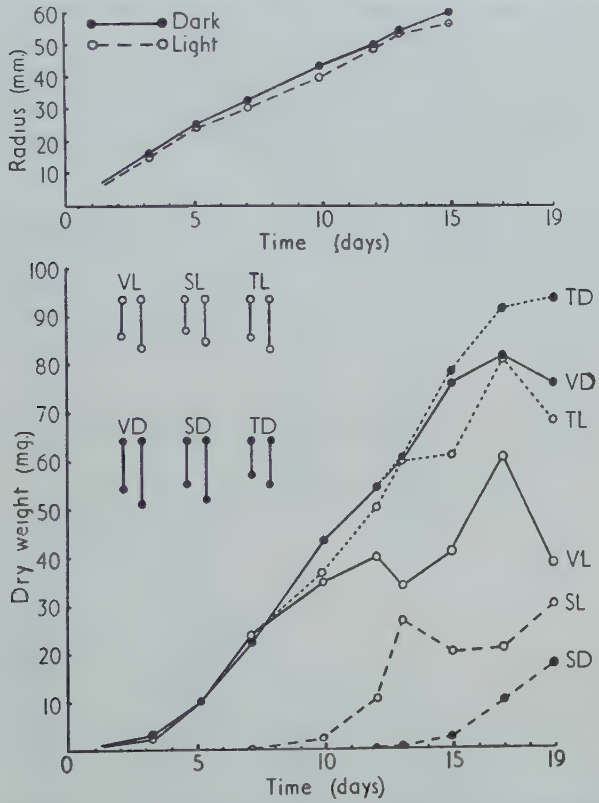
EXPERIMENTAL RESULTS

(*a*) *The effects of exposure to light*

Of a number of cultures on autoclaved horse dung in Petri-dishes, six were maintained in continuous illumination at about 8 foot candles while the rest were wrapped in black paper and kept in light-proof tins. At the first appearance of sporophore primordia in the illuminated series, a sample of three cultures was removed from darkness, taking care that no light was admitted to the remaining cultures. Observations on fruiting were made, and further samples of three taken at intervals.

It was found that sporophore primordia appeared in the illuminated series between the 8th and 11th days (mean of 9·5 days), but none were present in cultures taken from darkness until the 16th day when all in the sample bore initials.

In view of this behaviour, a comparison was made between growth in light and in darkness on agar media by following the progress of vegetative and reproductive growth gravimetrically, using a sampling technique. Cultures in medicine bottles were established from laterally placed inocula; half were incubated in the light and the rest were kept in darkness. Samples of four were removed simultaneously from each series at intervals over a period of 19 days, and the radii of the colonies and weights of mycelium and sporophore tissue determined.



TEXT-FIG. 1. The effects of light and darkness on radial growth of colonies (above) and growth in weight. *VL*, *SL*, and *TL* are respectively vegetative mycelium, sporophore tissue and total fungus for the series in the light; *VD*, *SD*, and *TD* for the series in darkness. The pairs of vertical lines in the lower diagram indicate the magnitude of significant differences between points on each graph at $P = 0·05$ (left line) and $P = 0·01$.

The results (Text-fig. 1) show that radial growth was virtually the same in both light and dark, and that fruiting in the light was approximately 5 days in advance of that in darkness. Although fruiting was delayed in darkness, the ultimate yield of sporophore tissue was similar to that in the light.

The bimodal graph for sporophore yield in the illuminated series corresponds to the production of two crops of sporophores, the second arising after the first began to autolyse. It will be noted that the production of each crop of sporophores in both light and darkness was associated with a fall in dry weight of vegetative mycelium.

In certain experiments reported below, gravimetric data on the effects of different light-treatments were obtained on only one harvesting date, usually about the 15th day after inoculation. In view of various results which indicated that the ultimate size of the sporophore yield was unrelated to the quantity of light received by the cultures, significant differences in sporophore yield on the 15th day were interpreted as indicating differences in the time at which fruiting had commenced, comparable to those shown in Text-fig. 1.

(*b*) *The quantity of light required to stimulate fruiting*

Preliminary experiments with cultures on various substrates indicated that a light exposure of only a few minutes duration at about the time fruiting would normally commence in continuous light was sufficient to stimulate fruiting. The minimal effective exposure was investigated in the following two experiments.

TABLE I

The Effects of Different Light Exposures on Growth and Fruiting of Coprinus lagopus

Fourteen days growth. Five replicates

Exposure (seconds)	Mean dry weight (mg.)		
	Mycelium	Sporophores	Total
0	69·2	4·2	73·4
1	52·0	22·7	74·7
5	53·3	20·4	73·7
10	50·1	26·0	76·1
60	49·8	25·3	75·1
180	41·7	29·9	71·6
300	45·1	27·7	72·8
Continuous	33·6	24·4	58·0
Signif. diff. between means $P = 0·05$	8·4	9·9	. .
Signif. diff. between means $P = 0·01$	11·4	13·4	. .

In the first, 9-day-old cultures taken from darkness were exposed to light from an incandescent filament bulb for periods ranging from 1 second to 5 minutes. The light intensity outside the medicine bottle at the level of the culture surface was 250 foot candles. The cultures were then returned to darkness for 5 days, after which growth and fruiting were recorded quantitatively (Table I). A one-second exposure was sufficient to accelerate fruiting; longer exposures had no significant further effect. In this experiment, as in certain others described below, the yield of vegetative mycelium from control cultures in continuous light was considerably less than in any other treatment. This effect is discussed later.

In the second experiment, ten-day-old cultures grown in darkness were exposed for the same period (5 seconds) to different light intensities and then returned to darkness for 5 days. The results (Table II) show that sporophore yield was significantly increased, compared with the treatment receiving no exposure, by a light intensity of 0·1 foot candles, and that higher intensities did not increase the yield further. However, cultures exposed to intensities of 0·1 foot candles or less lacked the profusion of primordia over the surface of the colony which was a feature of those exposed to brighter light. The mean yield of the treatment exposed to 0·01 foot candles was somewhat intermediate owing to differences among the responses of the replicate cultures.

TABLE II

The Effects of Exposures of Different Light Intensities on Growth and Fruiting of Coprinus lagopus

Fifteen days growth. Four replicates

Light intensity (foot candles)	Mean dry weight (mg.) Mycelium	Sporophores	Total
0	78·3	8·4	86·7
0·01	71·4	14·0	85·4
0·1	59·7	19·4	79·1
0·5	65·8	19·1	84·9
2·5	58·9	21·8	80·7
10	63·1	21·3	84·4
50	62·2	19·7	81·9
250	57·0	24·5	81·5
Continuous light	33·2	23·7	56·9
Signif. diff. between means $P = 0·05$	8·1	7·4	..
Signif. diff. between means $P = 0·01$	10·9	10·0	..

(*c*) *The onset of sensitivity to light*

To determine the age at which cultures became sensitive to light, a number of cultures on agar media were incubated in darkness, and at different times samples of four were removed and exposed to white light at 100 foot candles for 5 minutes. After exposure the cultures were returned to darkness. Those exposed at ages up to 11 days were examined and their yields harvested on the 14th day, while those exposed after the 11th day were left until the 20th day.

Table III shows that sensitivity to light developed on the 7th day or slightly later. The mean yield of sporophores from cultures exposed on the 7th day was lower than that of those exposed on the 9th primarily because two of the four replicates failed to respond to the exposure. The yield from cultures exposed on the 11th day was low owing to the short interval (3 days) between exposure and harvesting. Fruiting in cultures exposed on or after the 13th day was not significantly greater than in those kept in darkness.

(*d*) *Progress of the response to light exposure*

The behaviour of cultures during the period between their irradiation and the maturation of the subsequently formed sporophores, or the highest stage of their development attainable in the dark, was observed using a sampling procedure. Ten-day-old cultures were exposed for 5 minutes to white light at 250 foot candles and then returned to darkness, samples being taken subsequently at intervals. The mean yields of these samples and of cultures in continuous light and continuous darkness are given in Table IV. Primordia appeared within 27 hours of exposure, but several days elapsed before the yield of sporophore tissue became large, the sudden increase in sporophore tissue between the 15th and 16th days being accompanied by a highly significant fall in the dry weight of vegetative mycelium ($P = <0·01$).

TABLE III

The Effects of Light Exposures at Different Ages on Growth and Fruiting of Coprinus lagopus

(*a*) Harvested at 14 days old. Four replicates

Age at time of exposure (days)	Mean dry weight (mg.)		
	Mycelium	Sporophores	Total
3	80·2	0·2	80·4
4	75·9	0·1	76·0
5	84·7	0·1	84·8
6	83·8	0·3	84·1
7	75·9	9·1	85·0
9	63·8	18·4	82·2
11	75·7	5·2	80·9
Continuous darkness	81·9	1·5	83·4
Continuous light	42·7	28·4	71·1
Signif. diff. between means $P = 0·05$	11·7	7·9	..
Signif. diff. between means $P = 0·01$	15·9	10·7	..

(*b*) Harvested at 20 days old. Four replicates

Age at time of exposure (days)	Mean dry weight (mg.)		
	Mycelium*	Sporophores*	Total
13	50·0	26·7	76·7
16	50·9	24·8	75·7
Continuous darkness	57·2	21·2	78·4

* Treatment effects not significant at $P = 0·05$.

(*e*) *The effects of different light treatments on fruiting*

Since light affects the development of the individual fruit-bodies of *C. lagopus* (Borriss, 1934 *a*, *b*; Voderberg, 1950), it seemed possible that the *yield* of sporophore tissue might be influenced by the conditions of illumination after an exposure initiating fruiting. In the following experiment the effects of various light treatments, both before and after a standard exposure, were investigated, using cultures in Petri-dishes. The treatments are in Table V, which contains the results of this experiment.

On the basis of sporophore yield, the treatments fall into three groups, viz. (*a*) and (*f*) with the greatest yields; (*c*), (*d*), and (*e*) with somewhat lower yields, and (*b*) with practically none. The yields after a *particular* initial light stimulus (either initially continuous light or a brief standardized exposure) were thus virtually the same, irrespective of subsequent treatment, presumably owing to synchronization of fruiting. Plate XXII shows that the

TABLE IV

The Progress of Growth and Fruiting in Cultures of Coprinus lagopus *after Exposure to White Light*

Exposed to light 236 hours after inoculation

Age of sample (days)	No. of replicates	Mean dry weight (mg.)			Observations on fruiting
		Myc.*	Sporo.	Total	
10 (239 hr.) . .	5	36·0	0	36·0	No primordia
11 (263 hr.) . .	5	38·4	0†	38·4	All with primordia
12 (287 hr.) . .	5	55·0	0·1±0·07	55·1	Primordia enlarging
13 (311 hr.) . .	5	52·9	2·1±0·45	55·0	
14 (335 hr.) . .	5	55·8	6·1±1·04	61·9	Young etiolated sporophores
15 (362 hr.) . .	5	67·3	7·5±2·46	74·8	
16 (391 hr.) . .	5	53·3	25·4±2·60	78·7	‘Mature’ sporophores‡
16 (391 hr.) . . (continuous light)	4	31·9	22·9±4·56	54·8	Mature sporophores
16 (391 hr.) (continuous dark)	4	83·4	0·6±0·33	84·0	Few scattered primordia

* For comparison of two treatments, each with 5 replicates, significant difference at $P = 0{\cdot}05$, 7·8 mg.; at $P = 0{\cdot}01$, 10·5 mg. For comparison of a treatment with 5 replicates with one with 4, significant difference at $P = 0{\cdot}01$, 11·4 mg.

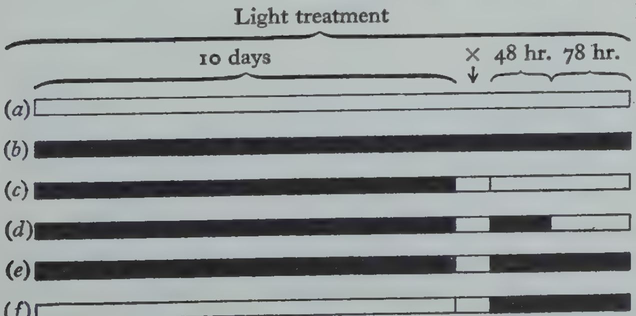
† Primordia of negligible weight.

‡ Sporophores full-sized, but mostly etiolated.

TABLE V

The Effects of Different Light Treatments on Growth and Fruiting in Cultures of Coprinus lagopus

Five replicates. X = 5-minute exposure at 250 foot candles



Light treatment	Mean final dry weight (mg.) Myc.	Sporo.	Total
(*a*)	52·0	27·0	79·0
(*b*)	86·4	0·3	86·7
(*c*)	65·6	21·0	86·6
(*d*)	63·0	19·9	82·9
(*e*)	66·8	17·5	84·3
(*f*)	54·6	32·2	86·8
Significant difference between means $P = 0{\cdot}05$	..	7·7	..
$P = 0{\cdot}01$	..	10·5	..

distribution of the sporophore material differed in these three groups. Cultures receiving initially continuous exposure (*a*, *f*) formed primordia, and ultimately several nearly mature sporophores, in an annular zone of the colony, while those whose first exposure to light was on the 10th day (*c*, *d*, *e*) all showed, in addition to primordia distributed as in (*a*) and (*f*), a more or less clearly defined ring of initials, presumably corresponding to the margin of the colony at the time of exposure. A feature of each of the five cultures maintained in continuous darkness (*b*) was the presence of circular patches of luxuriant mycelium which appeared to be growing over the adjacent normal portion of the colony (Pl. XXII (*b*)). These patches appeared in similar treatments in other experiments, but were never seen in cultures grown in continuous light.

(*f*) *The effect of different coloured lights on fruiting*

A number of 9-day-old cultures were exposed to lights of different colours. Control cultures were maintained in continuous white light. The exposures were standardized so that similar quantities of radiant energy were delivered at the level of the culture surface outside the medicine bottle. This was done with a photometer and a spectral response curve appropriate to the instrument. Allowance was not made for absorption by the glass wall of the culture vessel. The light intensities, as read by the photometer, were all adjusted to values equivalent to 2·5 foot candles for light through a filter with maximal transmission at 4,500 Å (blue). The colour filters used had a fairly wide band transmission, and are listed in Table VI by the wavelength at which transmission was maximal. Exposures of 5 seconds and 5 minutes

TABLE VI

The Effect of Light of Different Colours on Growth and Fruiting of Coprinus lagopus

Harvested at 14 days old. Four replicates

Light exposure		Mean dry weight (mg.)		
Colour	Duration	Mycelium	Sporophores	Total
Red	5 seconds	71·6	1·0	72·6
(6,600 Å)	5 minutes	67·2	2·4	69·6
Orange	5 seconds	74·4	1·0	75·4
(6,000 Å)	5 minutes	73·2	2·2	75·4
Yellow	5 seconds	67·5	3·1	70·6
(5,700 Å)	5 minutes	69·3	2·9	72·2
Green	5 seconds	70·8	2·3	73·1
(5,200 Å)	5 minutes	64·2	12·0	76·2
Blue-green	5 seconds	58·7	14·0	72·7
(4,950 Å)	5 minutes	61·2	13·7	74·9
Blue (4,500 Å)	5 seconds	56·1	15·5	71·6
White	Continuous	33·1	24·2	57·3
Signif. diff.	$P = 0·05$	7·5	4·1	. .
between means	$P = 0·01$	10·1	5·4	. .

were given at each wavelength except the shortest, at which 5 seconds alone was given, and 5 days later the response was measured gravimetrically. The cultures were kept at 25° C. throughout the experiment.

Table VI shows that fruiting was only stimulated by the shorter wavelengths. Green (5,200 Å) occupied a transitional position in that the fruiting yield was increased by a 5-minute but not by a 5-second exposure.

(*g*) *The distribution of the response to localized light exposure*

A number of experiments showed that sporophore primordia were produced after exposure to light only on mycelium in existence at the time of exposure. The following experiment was conducted to investigate whether there was any translocation of the light effect. The discovery that the fungus was insensitive to red light provided a means whereby colonies could be manipulated in conditions which did not affect fruiting.

Ten plate cultures on agar media were incubated in total darkness for 10 days, and then in red light a single disc, 17·5 mm. in diameter, was cut with a sterile cork-borer half-way along a radius of each colony, and transferred aseptically to an empty sterile Petri-dish. Five of the discs were then returned immediately to the cultures from which they were taken without further treatment, while the remaining five were exposed to daylight at approximately 50 foot candles for 15 minutes before they too were returned to the appropriate cultures. All cultures were then incubated in darkness for a further 5 days, after which the yields of vegetative mycelium and sporophores were determined separately for each disc and the rest of the colony.

TABLE VII

The Effects of Localized Light Exposure on Yield and Distribution of Sporophores in Cultures of Coprinus lagopus

Fifteen days growth. Five replicates

	Mean dry weight (mg.)						
	Disc			Rest of culture			Total fungus
Treatment	Myc.	Sporo.	Total	Myc.	Sporo.	Total	
Disc exposed to light . .	3·0 ±0·40	15·0 ±5·07	18·0 ±4·77	67·2 ±6·58	0·3 ±0·32	67·5 ±6·50	85·5 ±2·18
Disc not exposed	3·7 ±0·32	0·0	3·7 ±0·30	79·5 ±3·38	2·3 ±1·89	81·8 ±2·21	85·5 ±2·13

The results in Table VII show that fruiting was confined to the exposed discs, on each of which a tuft of sporophores had developed. On the rest of the colony, three of the five replicates of the exposed series had one to a very few small primordia of negligible weight. It was evident that organic continuity between the discs and the rest of the mycelium was re-established because the total weight of fungal tissue on the exposed discs was almost five times that on the unexposed, this being associated with a comparable reduction in total weight of the rest of the colony from which, presumably, materials were transferred.

Table VIII gives the result of a similar experiment in which whole halves of plate cultures were treated in a similar fashion to the above discs. Again the stimulation of fruiting was clearly restricted to the mycelium exposed to light. It will be noted that the mean yield of vegetative mycelium from the non-fruiting unexposed halves of the treated series of cultures was almost as low as that of the fruiting halves, and certainly much lower ($P = <0{\cdot}001$) than that in the controls, where the mean yields from both series of halves were similar. Since there was comparatively little difference between the mean total weights of cultures in the two treatments ($P = 0{\cdot}08$), fruiting in the exposed halves must have involved translocation of materials from the unexposed halves.

TABLE VIII

The Effects of Exposing Single Halves of Colonies of Coprinus lagopus *to White Light*

Sixteen days growth. Seven replicates

Treatment	Mean dry weight (mg.)						
	Unexposed or undisturbed half			Exposed or disturbed half*			Total fungus
	Myc.	Sporo.	Total	Myc.	Sporo.	Total	
One half exposed, other unexposed	27·9 ±2·43	0·0	27·9 ±2·42	19·2 ±1·58	37·2 ±3·08	56·4 ±2·17	84·3 ±0·96
Neither half exposed . .	44·5 ±2·80	3·1 ±1·67	47·6 ±2·54	43·0 ±1·41	0·7 ±0·46	43·7 ±1·28	91·3 ±3·24

* i.e. the half which was temporarily removed from the Petri-dish, and then exposed or kept in darkness.

(*h*) *The effect of temperature on growth and fruiting*

The temperature response of *C. lagopus* was studied in cultures on agar media which were exposed to daylight for at least several minutes each day. As has been shown above, exposures as brief as this stimulated fruiting as

TABLE IX

The Effect of Temperature on Growth and Fruiting of Coprinus lagopus

Fifteen days growth. Four replicates

Mean dry weight (mg.)	Temperature (°C.)						
	10	15–19	20	25	30	35	39
Mycelium . .	17·2 ±1·01	61·0 ±2·34	66·6 ±3·14	45·2 ±4·75	45·6 ±1·48	1·3 ±0·56	0
Sporophores .	0	0*	0·6 ±0·37	27·6 ±2·48	0	0	0
Total . .	17·2	61·0	67·2 ±2·84	72·8 ±2·90	45·6	1·3	0

* Primordia present, but of negligible dry weight.

effectively as continuous illumination. The temperature was not strictly controlled in the treatment labelled '15–19° C.', but was always within the limits indicated. The results of this experiment are given in Table IX, while in Table X are the results of a similar experiment conducted in complete darkness.

The general behaviour of the cultures was similar in both experiments. There was virtually no fruiting except at 25° C., and, as earlier experiments had shown, sporophore yield on the 15th day was considerably less in total darkness than in the light. Except for the effect of light, the response was essentially the same as that described by Voderberg (1949).

TABLE X

The Effect of Temperature on Growth and Fruiting of Cultures of Coprinus lagopus *in Continuous Darkness*

Fifteen days growth. Four replicates

	Temperature (°C.)						
Mean dry weight (mg.)	10	15–19	20	25	30	35	39
Mycelium . .	12·9 ±3·59	58·9 ±1·87	68·2 ±0·53	77·9 ±6·28	20·9 ±3·41	6·7 ±0·23	0
Sporophores .	0	0	0*	10·4 ±3·55	0	0	0
Total . .	12·9	58·9	68·2	88·3 ±5·46	20·9	6·7	0

* Primordia present, but of negligible dry weight.

TABLE XI

The Effect of Temperature on Fruiting of Coprinus lagopus *in Light and Dark*

	Intermittent light		Continuous darkness
Temperature (°C.)	Age at onset of fruiting (days)	Age at maturation (days)	Observations on 29th day
10	23	>29*	Each replicate with small primordia near sides of medicine bottle
15–19	13	24	Sporophores large, mostly etiolated
20	10	19	,,
25	8	13	,,

* Sporophores all less than 1 cm. high on 29th day.

In a number of fungi, fruiting is dependent on light only at low temperatures (Leonian, 1924). It therefore seemed possible that the partial dependence of *C. lagopus* on light at 25° C. might become a total dependence if the temperature were sufficiently low. Accordingly four replicate cultures were incubated at 10, 15–19, 20 and 25° C. in both intermittent light and continuous darkness for 29 days. During this period daily observations were made on the series in light, but the series in darkness were examined on only the final day.

Table XI shows that at none of the temperatures tested was fruiting prevented in darkness. The appearance of primordia was increasingly delayed as the temperature was lowered, and the period required for maturation of the sporophores lengthened, until at 10° C. it was not completed by the end of the experiment.

DISCUSSION

Contrary to Voderberg's (1949) finding, initiation of fruiting of *C. lagopus* in the experiments reported here was accelerated by light. It is possible that this difference in results may be due to differences in experimental technique, for unless a sampling procedure be adopted, the exposure to light when making observations is sufficient to stimulate the initiation of fruit-bodies owing to the exceptional sensitivity of the fungus. However, fruit-body initiation was not totally dependent on light for it ultimately occurred in darkness, at least between 10 and 25° C.

The response of *C. lagopus* to light was different from that of *Collybia velutipes* (Plunkett, 1953), for with this fungus the yield of sporophores depended on the duration of its exposure to light each day, the yield rising to a maximum as the daily period of illumination was lengthened. With *Coprinus lagopus*, however, the weight of fruit-bodies was affected by neither the duration of exposure nor the intensity of the light, provided these exceeded certain minima, nor by any subsequent sequence of light and dark.

The sensitivity of *C. lagopus* to light as a stimulus initiating fruiting is similar to the sensitivity of its fruit-bodies to light as a tropic and formative stimulus. Thus maximal phototropic sensitivity of the fruit-bodies is to light of wavelengths between 4,400 and 4,500 Å (dark blue) while there is virtually no response to light of wavelengths greater than 5,900 Å (orange). As a formative stimulus, light between 4,000 and 5,000 Å is very active while that of wavelengths over 6,400 Å evokes no response (Borriss, 1934*b*).

The data in Table III suggest that the period over which an adequate exposure to light will accelerate fruiting is approximately between the 7th and 13th days. Before this period, either the light-sensitive system has not developed or, alternatively, the effects of exposure cannot be retained until the colony is capable of response. In cultures exposed on the 10th day, the response is virtually immediate, being visible by the next day. On and after the 13th day it appears that the reaction which light exposure accelerates is completed, even in total darkness, for exposure exerts no further effect.

The stimulus was clearly not translocated to parts of the colony which were not exposed to light. Fulkerson (1955) states that the effect of light in stimulating pycnidium production by *Physalospora obtusa* is also not translocated. In certain experiments with *C. lagopus*, fruiting was confined by appropriate light treatment to single small discs of mycelium in the middle of the colonies, and materials were concentrated in those discs as a result of fruiting (Table VII). It seems more probable, therefore, that light exposure led to the production of a stimulant of fruiting rather than to the removal of an inhibitor, for it is likely that were the latter the case, the influx of intracellular materials from elsewhere would have introduced more of the inhibitor into the discs from the unexposed parts of the colony.

In several of the experiments reported above, the total weight of colonies about 15 days old was considerably less when grown in continuous light than when grown in darkness, or in darkness interrupted by exposure to light on about the 10th day. In the latter case, the cultures often bore similar yields of fruit-bodies to those in the light, but yielded appreciably more vegetative mycelium. Text-fig. 1 may suggest an explanation for these results. It shows that sporophore yield in continuous light changes relatively little after rising to its first peak about the 13th day, the subsequent loss through autolysis of the first crop of fruit-bodies being more or less counteracted by the development of a second. Also it is not until after the 13th day that the total yields for the series in the light begin to be appreciably less than those of cultures in the dark, no doubt due to this autolysis. The maintenance of a more or less uniform quantity of sporophore tissue in spite of autolysis must therefore place a continual drain on the reserves of the vegetative mycelium. Since sensitivity to light develops when cultures are about 7 days old (Table III), fruiting in continuous light may be 3 days in advance of that in cultures exposed only on the 10th day (cf. Table IV and Text-fig. 1). Thus when about 15 days old, cultures in the light will have already lost weight through autolysis (the loss being transferred to the vegetative mycelium) when briefly exposed cultures are attaining a similar level of fruiting for only the first time. Virtually non-fruiting cultures in continuous darkness will not, of course, be subject to such losses.

Text-fig. 1 shows that if fruiting was temporarily prevented simply by withholding light, growth in terms of radial extension and increase in dry weight continued at a uniform rate although conditions in the medium were favourable for fruiting, as was shown by the sporulation of the parallel series of cultures exposed to light. In this respect as in others (Madelin, 1956), *C. lagopus* does not conform to Klebs' (1900) principle that conditions favourable for fruiting are always more or less unfavourable for vegetative growth.

SUMMARY

Fruiting of *C. lagopus* in pure culture, which in continuous darkness commenced usually about the 15th day, was hastened by exposure to light between the 7th and 13th days of incubation. In continuous light fruiting generally commenced in 10 days.

Exposures to white light as brief as 1 second at 250 foot candles or 5 seconds at 0·1 foot candles were effective. Longer exposures, greater light intensities, or exposures followed by various light treatments did not produce significantly different yields of sporophores.

The response to exposure to light on the 10th day became evident as the formation of minute fruit-body primordia by the following day.

Only the blue range of the visible spectrum stimulated fruiting, the longest effective wavelength being near 5,200 Å.

The effect of light exposure was not translocated to unexposed portions of the colony.

A temperature near 25° C. was optimal for vegetative growth and fruiting in both light and dark. Fruiting ultimately commenced at all temperatures between 10 and 25° C., but was increasingly delayed at the lower temperatures where also the period for maturation was lengthened.

Acknowledgement

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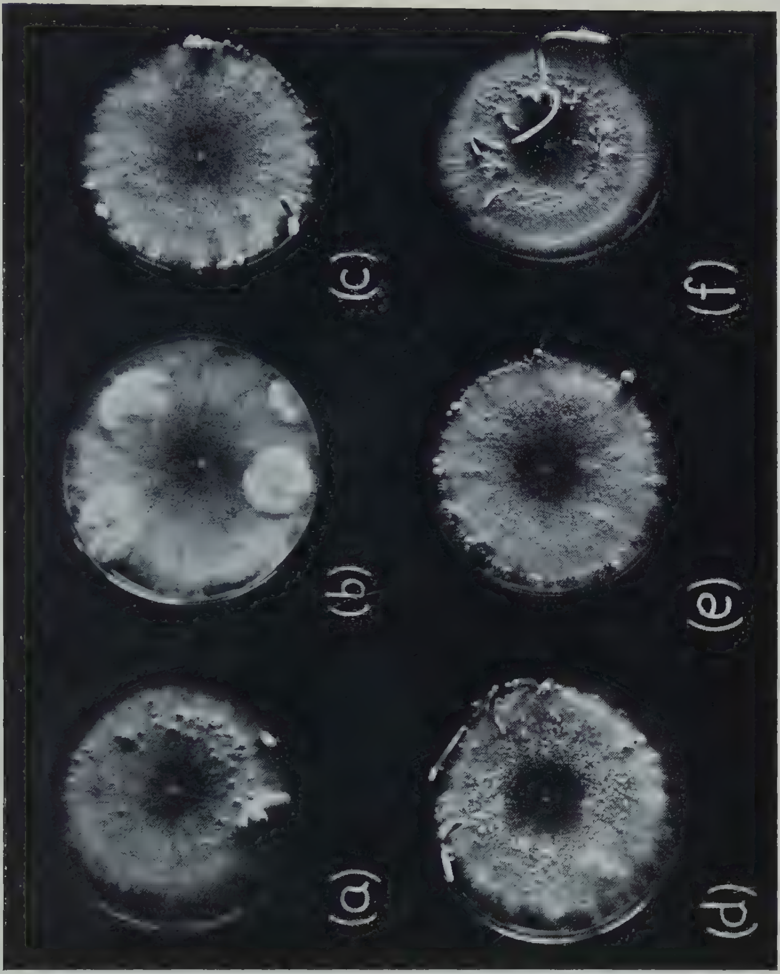
DESCRIPTION OF PLATE XXII

The effect of different light treatments on the production, distribution and development of fruit-bodies in plate cultures. A key to the six treatments is given in Table V in the text. Culture (*b*), grown in continuous darkness, shows patches of luxuriant mycelium.



M. F. MADELIN

Studies on the Germination of Cereals

2. Factors Determining the Germination Behaviour of Wheat Grains during Maturation

BY

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With four Figures in the Text

ABSTRACT

A comparative study has been made of the germination behaviour of a white-grained and a red-grained wheat variety throughout the course of maturation.

Although freshly harvested wheat grains do not germinate during the early stages of ripening, the embryo will develop under germination conditions when it is completely exposed, or the epidermis of the pericarp only is removed. At the harvest-ripe stage the white grains germinate rapidly, but the emergence of the embryo is considerably delayed in the red grains. The varietal difference is due to the presence of the covering layers and becomes increasingly apparent in grains which have been prematurely harvested, between 3 and 6 weeks after anthesis, and then subjected to desiccation. This effect of the covering layers on embryo development does not appear to be related to their permeability to water or oxygen. The behaviour of the two wheat varieties is thus determined by the mechanical properties of the covering layers, and especially the pericarp. These delay germination in the red grains until there has been an increase in the water absorbing power of the imbibed embryo.

INTRODUCTION

THE results of a previous investigation emphasized the importance of the stage of maturation reached by the wheat grain, in regard to its liability to germinate in the ear, which depends largely on the degree of desiccation. No grains would germinate while they were green, but with the disappearance of the chlorophyll, and the rupture of the layer containing chloroplasts during ripening, germination did occur. At this stage there was a difference between the grains of the white and red varieties, since the former, in general, germinated immediately, whereas the latter failed to do so until they had undergone further desiccation during after-ripening (Wellington, 1956). This varietal difference was not absolute, however, and a small proportion of the red grains germinated as soon as they were harvest-ripe, while the basal grains in the ears of the white variety remained dormant.

In this paper the internal factors which determine the different germination behaviour of the two varieties during maturation have been analysed. Several factors may be postulated: thus the permeability of the covering layers may affect the water or oxygen available to the embryo, and their tensile strength may limit the rate of embryo expansion. On the other hand desiccation may

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bring about changes in the embryo itself, during ripening and after-ripening, leading to more rapid development quite apart from any effect of the covering layers.

The effect of certain factors has been studied in the past in relation to the capacity of grains of different varieties to germinate at the harvest-ripe stage; but the part played by the factors enumerated above on the course of germination before this stage, and at stages between harvest ripeness and full maturity, has not been adequately investigated. Hiltner (1901) suggested that the ability to germinate resulted from the drying out of the grain which increased the permeability of the covering layers to water. But after a comprehensive survey of earlier work, Harrington (1923) concluded that the permeability of the covering layers to oxygen was probably the effective factor since all the treatments, by which germination had been induced, increased the availability of oxygen as well as water. Nilsson-Ehle (1914) had earlier reported differences in the structure of the covering layers in white and red grains which might account for the differences in their germination; while Hoyke (1938), experimenting with harvest-ripe grains, found that embryo development was more rapid after removal of the covering layers than when the grains were intact, and concluded that after-ripening had no direct effect on the embryo. He did not, however, compare the rate of embryo development with that of fully mature grains, although Nobbe and Hanlein (1877) had already suggested that changes might occur in the embryo of dormant seeds, during the rest period, which resulted in more rapid growth. As will be shown later, the rate at which the embryo develops, both in the intact grain and when the covering layers have been removed, is determined by the stage of maturation; and no assessment can therefore be made of the effect of the covering layers on embryo development unless the direct effect of maturation on the embryo is taken into account.

The present investigation therefore concerned not only the effect of the covering layers of white and red grains during the development, ripening, and after-ripening of the grain, but also the behaviour of the embryo under conditions in which the effect of the covering layers had been eliminated. The rate of embryo development was therefore studied in grains, at different times after anthesis, when all or some of the covering layers had been removed. In addition grains were removed from the ear at various stages before the normal time of harvest and subjected to desiccation; after which the growth of the embryo was observed when the covering layers were intact and wholly removed. Finally the behaviour of the embryo in the white and red grains at the stage of harvest ripeness was directly compared with that in similar grains at full maturity.

EXPERIMENTAL PROCEDURE

White grains of *Triticum vulgare* var. Holdfast and red grains of *Triticum vulgare* var. Atle were used for this investigation as their ability to germinate in the ear, at different stages of maturation, was already known. The stage of maturity reached by the grains of both varieties, at the time of testing, was

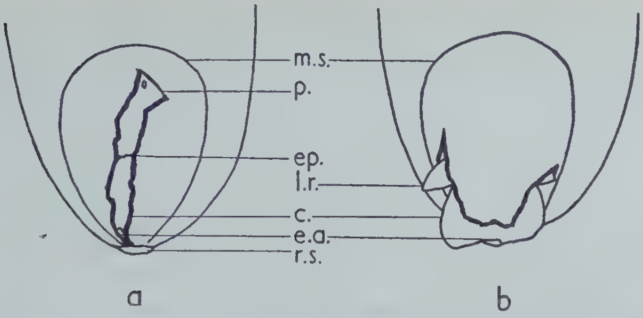

FIG. 1. The mode of rupture of the covering layers in a white grain (*a*), and a red grain (*b*); *c.*, coleorhiza; *e.a.*, embryonic appendage; *ep.*, epiblast; *l.r.*, lateral root; *m.s.*, margin of scutellum; *p.*, plumule; *r.s.*, rachilla scar.

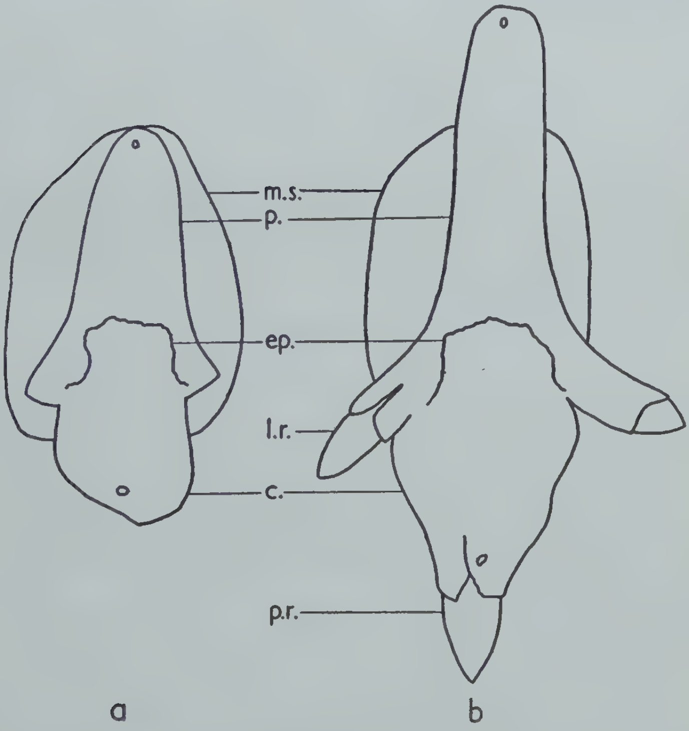

FIG. 2. Embryo development at the stage when the coleorhiza has started to expand (*a*), and the primary and lateral roots have started to elongate (*b*); *c.*, coleorhiza; *ep.*, epiblast; *l.r.*, lateral root; *m.s.*, margin of scutellum; *p.*, plumule; *p.r.*, primary root.

characterized by the period which had elapsed since the start of anthesis in the parent ear. In order to reduce differences in germination behaviour, due to the course of development and ripening in the ear, only the two basal grains from the 5th to the 7th spikelets on each side of the ear were used.

Germination took place under conditions of moisture supply which were optimal for fully mature grains and at a constant temperature of 16° C. The samples of grain were pooled and randomized, then lots of four were placed in each of the cells of 'spotting plates' with 0·2 ml. of water. The grains in six cells were used for each determination of embryo development; these were made at 24, 48, 72, and 120 hours after the start. Development was defined in terms of the number of embryos which had ruptured the covering layers when intact grains were used (Fig. 1); and by the number with the coleorhiza expanded and the coleoptile extending to the dorsal edge of the scutellum when the embryos had been exposed (Fig. 2*a*). In both cases the number of embryos with the primary root emerged and lateral roots elongating was subsequently determined (Fig. 2*b*); and in some experiments, the dry weight and moisture content was also determined after the embryos had been removed and dried at 98° C. for 24 hours.

EXPERIMENTAL RESULTS

1. *Changes in the Covering Layers and the Embryo during Development and Ripening of the Grains*

The origin and structure of the tissues which make up the covering layers have been described in detail by Percival (1921) and Pugh *et al.* (1932). The changes which occurred during the development and ripening of the grains were systematically observed in the course of the present work and these observations are summarized below.

The Epidermis (exocarp) retains its structure throughout, and while the cells are alive, appears to have a non-wettable surface. At first the cells contain starch, but this has disappeared at 3 weeks after anthesis, and from 3 to 6 weeks after anthesis irregular thickening is laid down on the lateral walls. Percival has reported that these cell walls eventually become 5–7μ thick and are perforated by pores which give a characteristic beaded appearance in surface view. During ripening the contents of the cells first become disorganized over the endosperm, but by the time the grain is harvest-ripe the cells over the embryo also appear to be dead.

The Sub-Epidermal Parenchyma (mesocarp) forms a loose tissue of large spherical cells which also contain starch during the early stages of growth. The expanding endosperm compresses this tissue, and at 5 to 6 weeks after anthesis, when the grain has reached its maximum volume, the cell lumina have disappeared.

The Green Parenchyma (endocarp) consists of an outer compact layer of rectangular cells (cross layer) with an inner layer of long irregular cells (tube cells) orientated at right angles. With the onset of ripening, at 5 weeks after anthesis, the chlorophyll disappears first from the chloroplasts in the cells over the endosperm and in the furrow. During the final stages of ripening the cross layer becomes disrupted and the cells collapse.

The Testa is apparent at 2 weeks after anthesis as a thin tissue enclosing a watery matrix representing the degenerate remains of the nucellus (Nutman, 1939) and the fluid contents of the embryo sac. The minute embryo at this stage lies in a pocket of the tissue opposite the micropyle. Two layers of square cells with no apparent contents were distinguished subsequently; the outer layer was covered by a cuticle which stained strongly in Sudan III. As the grain ripens the testa becomes more membranous and in the red variety an orange pigment appears in the cells.

The aleurone layer is already present at 2 weeks after anthesis as a layer of square cells with dense contents lining the whole of the embryo sac. Later, after the scutellum has been differentiated, the aleurone layer becomes firmly attached to its margin. During ripening the walls of the cells over the endosperm thicken but over the embryo the layer gradually becomes attenuated and finally collapses on to the testa.

When the sub-epidermal parenchyma collapses the covering layers become separated into two parts; the outer consists of the original epidermis, and the inner of the fused product of the green parenchyma of the pericarp, the cuticularized testa, and the aleurone layer. In the white grains the dual nature of the covering layers is more apparent during the shrinkage of the grain at 7 to 8 weeks after anthesis, as the epidermis is then thrown into folds over the dorsal region, whereas in the red grains both layers contract simultaneously and no folding of the epidermis takes place.

The behaviour of the epidermis in the two varieties at this stage indicates a difference in its mechanical properties which may subsequently affect the expansion of the embryo; because in the white grains there will be no resistance until the folds have disappeared, but in the red grains no swelling can take place without developing tension in the epidermis.

Marked changes also occur in the embryo during the development and ripening of the grain. Differentiation proceeds rapidly during the first 2 weeks after anthesis when the entire embryo appears to consist of meristematic cells arranged into zones representing the scutellum, plumule, and radicle. The embryo is attached to the testa at the micropyle by several large spherical cells forming an embryonic appendage (suspensor) similar to that described in the barley grain by Collins (1918). The coleoptile, epiblast, and coleorhiza are differentiated during the third week, by which time the embryo is complete, and starch appears in all the cells, except in the meristematic regions of the plumule and radicle. By the 4th week the cells forming the embryonic appendage constitute a wedge-shaped structure joining the embryo and the testa round the micropyle; and the contents of the cells have disappeared, being replaced in some cases by a dark brown substance. It is possible that this structure may play a part in the rupture of the covering layers, when the coleorhiza expands. There is little further change in the embryo, except for an increase in starch, until the final stage of ripening at 7 to 8 weeks after anthesis. The cells then contract, the tissues become less rigid, and all of the starch in the embryo rapidly disappears.

The changes in the dry weight and moisture content of the embryo are shown in Table I, and in a previous paper (Wellington, 1956) these have been related to similar changes in the grain as a whole. The dry weight of the embryo increases until the 6th week after anthesis, then remains constant during the 7th week, and falls slightly during the final stages of ripening. This is associated with a continuous reduction in the moisture content until the 5th week, after which it remains constant at about 120 per cent. until the rapid reduction which precedes harvest ripeness during the 8th week.

TABLE I

The Dry Weight and Moisture Content of the Embryo in Freshly Harvested Grains during Development and Ripening

Weeks after anthesis	Embryo dry wt. (mg.)		Embryo moisture content (per cent. dry weight)	
	White variety	Red variety	White variety	Red variety
3	0·20	0·26	296	333
4	0·78	0·78	161	193
5	1·17	1·22	122	122
6	1·62	1·58	112	114
7	1·61	1·52	111	122
8	1·43	1·47	10·9	12·9

2. *The Germination Behaviour of Immature Grains*

The rate at which the embryo developed under germination conditions was determined at weekly intervals from 3 to 7 weeks after anthesis. One series of tests was made with intact grains in order to determine the varietal differences which developed during normal ripening. A second series was made with grains from which the epidermis of the pericarp had been stripped in order to determine the effect of the inner covering layers which included the testa. A third series was made with grains from which a small portion of the covering layers, and the embryonic appendage, had been removed at the micropyle in order to ensure free access of water and oxygen to the embryo. Finally, a control series of tests was made with grains from which the covering layers over the embryo had been entirely removed in order to determine whether its rate of development was directly influenced by the stage of ripening.

The detailed results of these tests are shown in Fig. 3 which gives the number of embryos out of a total of 24, which had emerged or had started to develop after 24, 48, 72, and 120 hours respectively. In addition the number in each treatment with the primary root emerged is shown by the black portion of the histograms. The results for the white and red varieties are presented separately, and indicate the behaviour of the embryos in each treatment at weekly intervals.

Intact grains: No visible development of the embryo occurred in either type of grain until the onset of ripening at 5 weeks after anthesis and less than half had emerged after 120 hours at 6 weeks. By 7 weeks after anthesis the embryos in the white grains were able to emerge early in the test, and the majority had reached the stage of root development at 120 hours, when less than half of the embryos in the red grains had even emerged. In the white grains the embryo usually emerged after a split had developed in the covering layers which simultaneously exposed the plumule, epiblast, and coleorhiza (Fig. 1*a*); whereas in the majority of the red grains the covering layers split across the lower part of the embryo and the coleorhiza emerged first (Fig. 1*b*). In some of the red grains the epidermis remained intact after the inner layers had been ruptured by the elongation of the plumule.

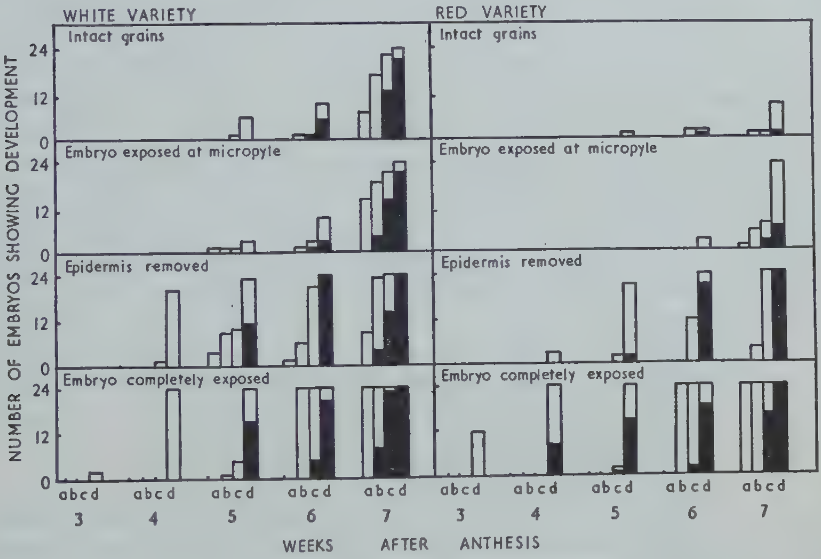

FIG. 3. Embryo development in germination tests on freshly harvested grains during development and ripening (3 to 7 weeks after anthesis); □ embryos with coleorhiza expanded; ■ embryos with roots elongated; *a*, after 24 hours; *b*, after 48 hours; *c*, after 72 hours; *d*, after 120 hours.

Embryo exposed at micropyle: Embryo development was very similar to that in the intact grains of both varieties until 7 weeks after anthesis. At this stage the number of the embryos in the red grains which had emerged at 120 hours was greater in this treatment, although the rate of development still did not approach that of the embryos in the white grains.

Epidermis removed: In both varieties the embryos swelled rapidly and the inner covering layers were soon split by the elongation of the plumule which then emerged followed by the epiblast and coleorhiza. In contrast to the previous treatments, the majority of the embryos in both varieties had emerged in 120 hours at 5 and 6 weeks after anthesis, and a considerable number in the white variety were able to do so at 4 weeks. The rate of embryo development in the white grains at 7 weeks was the same as in the previous treatments; but in the red grains it was considerably increased and all of the embryos had emerged at 72 hours.

Embryo completely exposed: As ripening proceeded there was a progressive reduction in the time which elapsed before the embryo started to develop when the covering layers had been removed; and the difference between the rate of development of the embryos in the white and the red varieties was also eliminated by this treatment at all stages. At 3 weeks after anthesis the coleorhiza of relatively few embryos had expanded at 120 hours, but at 4 and 5 weeks all had reached this stage. With the onset of ripening there was a marked increase in the rate of development, and the time required for expansion of the coleorhiza was reduced to 48 hours at 6 weeks and 24 hours at 7 weeks.

There was thus a direct effect of ripening on the ability of the embryos to develop under optimal germination conditions, when exposed, and this increased up to the harvest-ripe stage. The presence of the inner covering layers alone did not greatly retard development at any stage, but when the epidermis also was present, the embryo was incapable of development as long as the grains were green (indicating that the cells in the covering layers were still alive) even when there was free access of water and oxygen at the micropyle. However, as soon as the cells had died during ripening, the embryos were capable of rapidly overcoming the constraint imposed by the covering layers in the white variety, but not in the red.

3. *The Effect of Premature Desiccation*

An experiment was carried out in which the embryo development was determined, at a standard time of 8 weeks after anthesis, using as controls grains which had attained harvest ripeness under normal conditions. These were compared with samples which had been harvested at weekly intervals from the 3rd week onwards and then dried for the rest of the 8-week period. In this way the changes in the covering layers associated with ripening were induced at different stages of grain development, and a comparison of the behaviour of the pre-desiccated grains with those harvested at comparable times after anthesis and put to germinate immediately (Fig. 3) provides a measure of their effect. In order to determine whether there was a direct effect of desiccation on the embryo, tests were also made on grains with the embryo exposed, except in the case of the grains harvested 3 weeks after anthesis which were so shrivelled that the embryo could not be exposed without damage.

The grains were taken from ears of both varieties, harvested at weekly intervals from 3 to 7 weeks after anthesis, and then kept in an atmosphere of 50–55 per cent. relative humidity. The dry weight and moisture content of these embryos at the time of testing are shown in Table II, from which it will be seen that in all of the grains the moisture content of the embryo was reduced by this treatment to approximately the same level as in harvest-ripe grains. The fact that the dry weights were in general lower than those in Table I was probably due largely to the difficulty of removing the entire embryo when the grains were dry.

TABLE II

The Dry Weight and Moisture Content of the Embryo in Grains after Premature Harvesting and Drying

Time of harvest (weeks after anthesis)	Period of desiccation (weeks)	Embryo dry wt. (mg.)		Embryo moisture content (per cent. dry weight)	
		White variety	Red variety	White variety	Red variety
3	5	0·40	0·48	5·0	1·0
4	4	0·87	0·92	10·6	5·0
5	3	1·22	1·33	8·9	5·6
6	2	1·15	1·27	14·1	5·3
7	1	1·45	1·47	8·9	5·1

The detailed germination results are shown in Fig. 4. The data for the white and red varieties are presented separately; and for each variety the behaviour of the intact grains, and those with the embryo exposed, are also shown. The results shown in Fig. 4 may be directly compared with those for corresponding samples shown in Fig. 3, the only difference being that in the former case the grains have been given an additional period of desiccation.

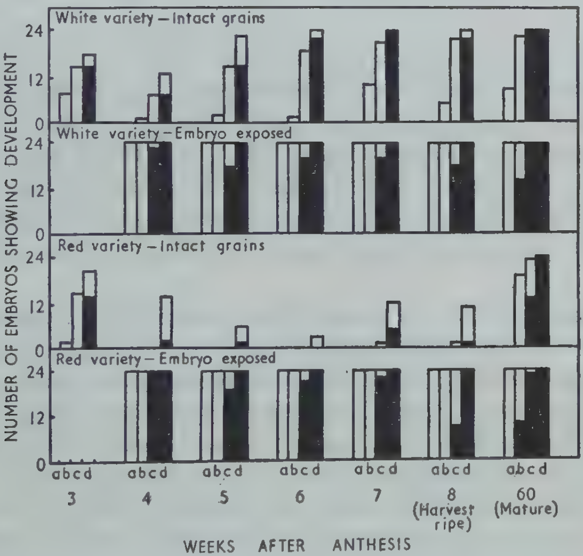

FIG. 4. Embryo development in germination tests on grains after premature desiccation, and at the harvest-ripe, and fully mature stages; □ embryos with coleorhiza exposed; ■ embryos with roots elongated; *a*, after 24 hours; *b*, after 48 hours; *c*, after 72 hours; *d*, after 120 hours.

Intact grains: The embryos in the grains harvested at 3 and 4 weeks, and allowed to dry down, showed considerable development at 120 hours, unlike those in the freshly harvested grains. There was no difference between the two varieties in the samples taken at 3 weeks after anthesis but in the samples taken later, however, very marked varietal differences in germination behaviour appeared. In the white variety development was somewhat slower in the 4-week sample, but was again more rapid in that harvested at 5 weeks, and remained substantially unchanged in later samples up to the harvest-ripe stage at 8 weeks. By contrast, germination of the red grains was greatly reduced in the sample harvested at 4 weeks, and was further reduced, reaching a minimum value, in the sample taken at 6 weeks. The sample taken at 7 weeks, however, germinated as readily as that of the normally harvest-ripe grains; but the development of the embryos was far below that in white grains at the corresponding stage.

Embryo exposed: Premature desiccation, at all stages, resulted in a marked increase in the rate of development of the embryo when the covering layers had been removed. The differences between the two varieties disappeared and embryo development reached the same level in the grains which were harvested and dried at 4 to 7 weeks after anthesis, as in those which had ripened under normal conditions and were harvested at 8 weeks. In all cases the initial stage of embryo development, depicted in Fig. 2*a*, was completed in 24 hours.

Premature desiccation therefore had a direct effect on the behaviour of the embryo which was independent of its dry weight or the stage of development when desiccation occurred; this effect was to increase the rate of development to that of the embryos in harvest-ripe grains. And as subsequent investigation showed that this treatment also resulted in the complete disappearance of starch from the embryo, it seemed possible that the increased rate of development might be associated with a higher osmotic pressure following starch hydrolysis.

The complete inhibition of germination, caused by the covering layers in the early stages of grain development, was also removed by premature desiccation. This was true of both the white and the red variety and the striking fact emerges that desiccation of intact red grains, at 3 weeks after anthesis, allowed more development to take place than occurred even at the harvest-ripe stage.

The beneficial effect of desiccation in the intact red grains rapidly disappeared when it was applied at later stages of grain development, reaching a minimum at 6 weeks after anthesis, at which time the thickening of the epidermal walls was complete. This failure to germinate in the pre-treated grains (even more apparent in the intact grains not subjected to desiccation) cannot be attributed to direct inhibition of the embryo; for exposure leads at once to rapid development at all stages. On the contrary, to account for the fact that pre-desiccation increases the growth-rate of exposed embryos to the level at the harvest-ripe stage, it would be necessary to postulate the production of a growth accelerator rather than the progressive accumulation of an inhibitor, which might be inferred from the germination behaviour of the intact red grains after pre-treatment. Moreover, the rapid germination after pre-desiccation at 3 weeks is further evidence against the view that an inhibitor is concerned. The different capacity of the grains to germinate during ripening cannot therefore be attributed to a direct inhibition of the embryo but is due to the effect of the covering layers.

4. *The Effect of the Covering Layers on the Embryo Development in Harvest-ripe and Fully Mature Grains*

In germination tests on harvest-ripe and fully mature grains of both varieties the moisture content and dry weight of the embryos were determined at intervals in addition to their development. The harvest-ripe grains were taken from ears harvested at 9 weeks after anthesis, with an embryo moisture content of 17 per cent.; while those which were fully mature were taken from ears which had been stored for a year under dry conditions (50–55 per cent. relative humidity) and had an embryo moisture content of 8 per cent. As in the previous experiments, duplicate tests were made on grains with the covering layers intact and with the embryo exposed; and the development, moisture content, and dry weight of the embryos in both treatments are shown in Table III and Table IV respectively. All the determinations were based on replicate samples of 24 grains.

TABLE III

The Development, Moisture Content, and Dry Weight of the Embryo in Harvest-ripe (HR) and Mature (M) Grains with the Covering Layers Intact

		White variety				Red variety			
Germination period (hours)	Stage of maturity	Embryo emerged	Roots emerged	Moisture content (per cent. dry weight)	Dry weight (mg. per embryo)	Embryo emerged	Roots emerged	Moisture content (per cent. dry weight)	Dry weight (mg. per embryo)
24	HR	0	0	134	1·54	0	0	114	1·59
	M	9	0	129	1·76	0	0	115	1·74
48	HR	15	0	162	1·67	0	0	123	1·56
	M	23	0	237	1·72	19	0	196	1·76
72	HR	21	11	236	1·74	0	0	136	1·34
	M	24	24	361	2·22	23	13	309	2·11
120	HR	24	24	507	3·09	21	1	258	1·55
	M	24	24	588	4·36	24	24	595	4·05

Intact grains: At 24 hours the covering layers of the harvest-ripe white grains were still intact, although the folds in the epidermis had disappeared, whereas the covering layers of some mature grains were already ruptured; the harvest-ripe and mature red grains were all intact at this stage. The moisture content of the embryo in both harvest-ripe and mature grains had reached approximately the same level at 24 hours; although it was somewhat higher in the white variety than in the red. At 48 hours, the embryo had emerged in the majority of the mature grains of both varieties and in more than half of the white grains which were harvest-ripe; no development had yet taken place, however, in the harvest-ripe red grains, nor had there been any further increase in the moisture content of their embryos. At 72 hours, the embryos in all the mature white grains, and half of the mature red grains, had reached the stage of root development shown in Fig. 2*b*; and at this stage the further increase in moisture content was associated with an increase in the embryo dry weight. Root elongation had also started in the white grains at the harvest-ripe stage although the embryos had not yet started to increase in dry weight; but the red grains still showed no signs of development, except for a slight increase in the embryo moisture content. At 120 hours the embryos in the mature grains of both varieties, and in the harvest-ripe grains of the white wheat, had all completed the initial stages of seedling development. By this time the embryos in the harvest-ripe red grains had also emerged, but their moisture content was still much lower, and the dry weight of the embryo had not yet increased nor had the roots elongated. This lag in the development of the embryo in the harvest-ripe red grains was associated thus with the long delay in rupturing the covering layers.

TABLE IV

The Development, Moisture Content, and Dry Weight of the Embryo in Harvest-ripe (HR) and Mature (M) Grains with the Embryo Exposed

		White variety				Red variety			
Germination period (hours)	Stage of maturity	Coleorhiza expanded	Roots emerged	Moisture content (per cent. dry weight)	Dry weight (mg. per embryo)	Coleorhiza expanded	Roots emerged	Moisture content (per cent. dry weight)	Dry weight (mg. per embryo)
24	HR	24	0	153	1·48	24	0	137	1·47
	M	24	0	186	1·65	24	0	195	1·56
48	HR	24	11	269	1·70	24	0	231	1·50
	M	24	14	299	1·79	24	10	303	1·83
72	HR	24	21	375	1·84	24	15	304	1·47
	M	24	24	427	2·45	24	23	452	2·45
120	HR	24	24	545	3·69	24	24	555	3·08
	M	24	24	680	4·69	24	24	605	4·45

Embryos exposed: Removal of the covering layers again eliminated all varietal differences in embryo development (Table IV). The rate of water uptake by the embryos was greater when they were exposed than in the intact grains; and there was also a consistent difference in favour of the embryos in the fully mature grains. This difference between the exposed embryos in mature and harvest-ripe grains in respect to water uptake suggests that it is not the permeability of the covering layers alone which is the controlling factor; and it will be noted that there was no such difference in the moisture content of the embryos in the intact grains at 24 hours. Since the moisture content of the exposed embryos may be regarded as a measure of the water-absorbing power of the embryo tissues, this is clearly greater in the mature than in the harvest-ripe embryo.

The moisture content of the exposed embryos increased from 17 per cent. to 137 per cent. in the harvest-ripe red grains and from 8 per cent. to 195 per cent. in the mature grains during the first 24-hour period, and in both cases development had reached the stage of expansion of the coleorhiza; whereas in the same period the embryo moisture content in the intact harvest-ripe and mature grains had increased to 114 per cent. and 115 per cent. respectively without any development of the embryo taking place. Thereafter the embryo moisture content in the intact harvest-ripe grains increased slowly to 136 per cent. at 72 hours, while in the mature grains it reached 309 per cent.; in the former case no development had taken place, whereas in the latter the development of the embryos was almost completed. This increased rate of water uptake in the mature grains was associated with the rupture of the covering layers, between 24 and 48 hours, which in the harvest-ripe grains remained intact after this time. And it is suggested that the very slow water uptake by the embryo in the harvest-ripe grains was related to the failure of the covering layers to split.

It will be shown in a subsequent paper that water uptake by the embryo occurs in well-defined phases, the first being rapid uptake by imbibition which restores the embryo to its original volume, before the final stage of desiccation during ripening, when the embryo moisture content is about 120 per cent. (Wellington, 1956). The initial rapid phase is followed by a slow increase, during which time emergence of the embryo occurs in the white grains, and then by a second phase of rapid uptake which is associated with cell vacuolation and growth. The imbibitional phase is completed while the covering layers are still intact, and in the red grains there is then a slow increase in moisture content corresponding to the expansion of the embryo up to the elastic limit of the covering layers. It would appear that there is an increase in the water-absorbing power of the embryo during the after-ripening period (Table IV); and so the force exerted by the expanding embryo in the mature grains is sufficient to rupture the covering layers much more rapidly than in the harvest-ripe grains. It is also significant that the embryo moisture content in the harvest-ripe red grains, which heralds the splitting of the covering layers at 72 hours, is 136 per cent. and is the same as that in the white grains (134 per cent.) which precedes splitting at 24 hours.

5. *The Effect of Increased Water-supply by Direct Exposure of the Endosperm*

The final experiment which was carried out consisted of removing a quarter of the endosperm, and the associated covering layers, from the distal end of grains of the red variety at the harvest-ripe stage; and then germinating them with the cut surface in water. The development of the embryo in the grains which were treated in this way is shown in Table V, and their embryo moisture content and dry weight is shown in Table VI, together with that of grains with the embryo exposed, and with the covering layers intact.

Under the conditions of this experiment the supply of water to the embryo was quite independent of the permeability of the covering layers to water, and the moisture content of the embryos at 24 hours was similar to that of the exposed embryos, reaching the same level as that in the intact grains prior to emergence. The outstanding point of difference is the fact that between 24 hours and 72 hours the embryo moisture content only increased from 114 per cent. to 136 per cent. in the intact grains, whereas in the grains with the distal end removed it increased from 140 per cent. to 354 per cent. over the same period, and in this connexion the behaviour of the covering layers is of interest.

TABLE V

Embryo Development in Grains of the Red Variety at the Harvest-ripe Stage with the Distal End Removed

Germination period (hours)	Covering layers intact	Epidermis only intact	Coleorhiza expanded	Roots Emerged
24	24	0	0	0
48	7	5	12	0
72	7	2	15	3
120	0	(2)*	22	15

* The lateral roots of the embryos had elongated beneath the epidermis in both of these grains.

While no splitting occurred in the intact harvest-ripe grains (Table III), 12 of the embryos in the 24 grains with the distal end removed behaved in a similar way to those in the intact mature grains, with rupture of the covering layers at 48 hours. Of the remainder, 5 showed the inner layers split but not the epidermis (Table V); in these the plumule had broken through the inner layers, and the lateral roots, which had also elongated, remained coiled under the epidermis. The embryos in the remaining 7 grains had still not ruptured the covering layers at 72 hours. Meanwhile the moisture content of all the embryos was increasing rapidly (Table VI), showing that water uptake was not impeded in any way when the distal end of the grain was removed.

TABLE VI

Embryo Moisture Content and Dry Weight in Grains of the Red Variety at the Harvest-ripe Stage with the Distal End Removed (A), *with the Embryo Exposed* (B), *and with the Covering Layers Intact* (C)

Germination period (hours)	Embryo moisture content (per cent. dry weight)			Embryo dry weight (mg. per embryo)		
	A	B	C	A	B	C
24	140	137	114	1·66	1·47	1·59
48	266	231	123	1·70	1·50	1·56
72	354	304	136	2·01	1·47	1·34
120	445	555	258	2·66	3·08	1·55

In the case of the intact grains it has been suggested that water uptake was impeded by the back pressure of the covering layers after the initial stage of imbibition had been completed, but when the distal end of the grain was removed this constraint was not present. The reason for this is that the embryo could now expand fully by pushing the endosperm out of the cut end of the grain. That this expansion was not less than that of the exposed embryos may be inferred from the similar rate of water uptake in both cases (Table VI).

The entry of water through the distal end also facilitated the hydrolysis of reserves in the endosperm which was reflected by the earlier increase in the dry weight of the embryo in these grains than when the embryo alone was exposed. After imbibition was completed, the water-absorbing power of the embryo would depend on the osmotic pressure of its cells which in turn would depend on the transfer of soluble material from the endosperm. This is probably the reason why the embryo moisture content of the grains with the endosperm exposed was higher at 48 and 72 hours than that of the grains with the embryo exposed.

DISCUSSION

The results of these experiments may now be considered in relation to the factors which have already been postulated to account for the different germination behaviour of the white and red varieties during maturation.

The availability of water and oxygen to the embryo

The availability of water was controlled by partial or complete removal of the covering layers over the embryo, and in the harvest-ripe red grains, by the exposure of the endosperm leaving the covering layers over the embryo intact. The inhibition of germination in the red grains at the harvest-ripe stage is the outstanding varietal characteristic, and at this stage water absorption by the embryo was equally as rapid when water entered freely through the endosperm, as when the embryo was directly exposed, whereas water absorption by the embryo in the intact grains was much slower (Table VI). During the initial phase of uptake, which is attributed to the imbibition of the colloids of the embryo, the moisture content of the latter increased rapidly in the first 24 hours; but when this stage was completed further uptake remained slow until after 72 hours (Table III). When water was supplied directly to the exposed embryos, or through the endosperm, the second phase of rapid uptake followed immediately, whereas in the intact grains it did not begin until the covering layers had been ruptured. This retardation of water uptake is not due to any property of the embryo, as exposure has shown, but must be due to the covering layers. And in this respect the harvest-ripe white grains behaved quite differently; for in them there was no retardation because soon after the water content of the embryo had reached the level of full imbibition (120 per cent.) rupture of the covering layers occurred. It would appear, therefore, that water-supply is not a determining factor in the varietal difference.

Taylor (1942) has shown that a supply of oxygen is necessary for the full development of the embryo during the germination of the wheat grains; and this is confirmed by the fact that the coleorhiza does not expand when grains with the embryo exposed are completely immersed in water. But since this stage was initiated in the harvest-ripe red grains by removing the distal end, without altering the conditions for oxygen diffusion to the embryo (Table V), a varietal difference in the permeability of the covering layers to oxygen would not adequately account for the different germination behaviour at harvest ripeness. The complete inhibition of germination, while the grains were green, may have been due partly to the impermeability to oxygen of living tissues in the covering layers, as suggested by Kidd and West (1920). But the development obtained by removing only the epidermis indicates that this effect is not due to the tissue containing chloroplasts; and the apparent correlation between the disappearance of chlorophyll and the increased capacity for germination is probably an indication of the more general effect of desiccation in killing all of the cells in the covering layers. Moreover, embryo development in grains with the embryo exposed at the micropyle remained the same as in the intact grains; and the slight effect of this treatment on the red grains at the harvest-ripe stage could be attributed to the weakening of the covering layers by the operation rather than to the enhanced supply of oxygen. The effect of oxygen tension has been studied subsequently and the results confirm that it is not a major factor in the delayed germination of wheat.

The mechanical properties of the covering layers

The causal factor in the varietal difference, which resides in the covering layers, is more likely to be of a mechanical nature resembling in its effect that reported in Johnson Grass by Harrington and Crocker (1923). The presence of pigment in the testa of the red grains does not appear to have a direct effect on germination, as it is present after desiccation at all stages of maturity. It may, however, be associated with other anatomical differences (Nilsson-Ehle, 1914); such as, for example, the condition of the covering layers at harvest ripeness, when the white grains have a wrinkled surface and the red grains are smooth. During the first stage of water uptake swelling of the embryo in the white grains merely removes the wrinkles without occasioning any tension in the epidermis, while in the red grains increasing tension must result. Furthermore, the rupture, when it occurs, takes a different form in the two varieties; the split in the covering layers of the white grains simultaneously exposes both the plumule and coleorhiza, as against the split in the region at the base of the embryo in the red grains which leads to emergence of the coleorhiza first. This behaviour of the covering layers must be due to some difference in their mechanical properties. The data in Fig. 3 demonstrate clearly that of the covering layers it is the epidermis which is mainly concerned, since in both white and red grains removal of this tissue leads to more rapid expansion of the embryo at all stages, and the effect of its removal approaches that of direct exposure of the embryo.

The epidermis in the red grains appears to react in the same way as rubber which under increasing tension at first elongates like a truly elastic substance up to a limit when elongation ceases and rupture occurs. Thus the pressure exerted by the imbibed embryo leads to a limited extension of the epidermis, then until rupture occurs, water uptake almost ceases. When the rupture occurs depends upon the water-absorbing power of the embryo; and this in turn will depend upon the osmotic force which can be exerted after imbibition.

The increased tension developed in the epidermis by the osmotic forces of the embryo may arise in two ways, either by absorption of carbohydrate from the endosperm in the form of sucrose, or by the hydrolysis of the starch which is present in the embryo in the early stages. It has been observed that the starch in the embryo disappears during normal ripening, in the 7th and 8th weeks after anthesis, when the intact grains in both varieties show an increased ability to germinate. In particular the sudden disappearance of starch from the coleorhiza is associated with the rapid swelling of this tissue, if water is available, which provides a mechanism for rupture.

In the normal developing grain desiccation proceeds slowly in the earlier stages, being associated with the thickening of the epidermis and the disappearance of the green colour. These slow changes have been hastened by drying down the grain, at various stages of development, to a moisture content similar to that of the harvest-ripe grain. When this is done the importance of the stage of development to germination capacity is readily apparent. At 3 weeks from anthesis when neither variety will germinate if freshly harvested, this treatment leads to rapid embryo development with no varietal differences; but at later stages marked differences appear between the two varieties. The white grains show a small minimum at 4 weeks and then an increased capacity to germinate which is substantially unchanged up to the harvest-ripe stage. By contrast pre-desiccation of the red grains causes increasing retardation of germination up to 6 weeks (Fig. 4), after which there is some increase in capacity to the harvest-ripe stage, but this is always much less than in the white grains. It is during this period of development, therefore, that the characteristic varietal differences develop; since desiccation at 3 weeks is equally effective in both varieties, it must be assumed that the particular characteristic of the covering layers of the red variety is not yet manifest, and observation has shown that the thickening of the epidermis has not yet begun at this stage. It can be further seen from the data in Fig. 4 that the subsequent effect of the stage of development is not associated with the embryo itself, because in both varieties exposure of the embryo leads to extremely rapid development.

The progressive changes internal to the grain

It is evident from the data for the red variety in Fig. 4, which shows a definite minimum in germination at 6 weeks after anthesis, that there must be two opposing factors concerned; one which tends to reduce the germination capacity and is probably due to the increasing tensile strength of the epidermis, and the other which tends to increase the germination capacity by increasing the osmotic force exerted by the embryo. As already mentioned, this increased osmotic force may be due either to absorption of carbohydrate from the endosperm, or to hydrolysis of starch *in situ* in the embryo. The dry-weight data in Table III show that it is the latter and not the former source of carbohydrate which is effective in the intact grains. Desiccation may in this case, as in the wilting leaf, accelerate the hydrolysis of starch and this would account for the fact that when the rate of desiccation during normal ripening proceeds rapidly after the 6th week, the rate at which germination takes place in the intact grains also increases. This view is confirmed by the behaviour of the exposed embryos in the pre-desiccated grains, which are characterized at all stages both by an apparent absence of starch, and by a rate of development equal to that of embryos exposed at the harvest-ripe stage. Further desiccation after the harvest-ripe stage has already been shown to increase germination capacity, particularly in the case of the red variety (Wellington, 1956); and the reduction in moisture content from 17 per cent. to a minimum of 8 per cent., which occurred when complete maturity was reached after dry storage, was associated with an increase in the water absorbing capacity when the embryos were exposed (Table IV). It is, of course, possible that the enhanced capacity for germination, as a result of 'after-ripening', may be due to some change in the covering layers, and so far this has not been experimentally investigated. However, it has been shown by Brown (1940) and others that with certain seed coats drying decreases the permeability to oxygen and it seems unlikely, therefore, that permeability changes can account for the behaviour noted.

The failure of the intact grains to germinate when harvested in the early stages of development may possibly be due to the presence in the grain of an inhibitor. This seems improbable as grains of both varieties harvested at 3 weeks after anthesis readily germinate after pre-desiccation. And as the inhibition is most marked at 6 weeks in the red grains, and to a less extent at 4 weeks in the white grains, any postulated inhibitor would have to increase as development proceeds. The evidence, however, is against the view that an inhibitor is concerned because its removal should result only in development as rapid as that at the time of greatest growth potentiality in the normal grains; whereas pre-desiccation results in more rapid growth of the exposed embryos than at any stage in the normal grains up to harvest ripeness.

It seems more probable that the delay in germination of the red grains is due to the constraint imposed by the intact epidermis being greater than in the white grains. This can only be overcome by the embryo when the pressure it exerts by its osmotic force has reached a maximum, as it does when the moisture content of the grains is reduced to 8 per cent. during after-ripening, or when access of water through the distal end of the harvest-ripe grains leads to more rapid hydrolysis of starch and consequent increased sugar supply to the embryo. The weight of the evidence therefore appears to conform with the view that the varietal difference in germination is caused by the different mechanical properties of the covering layers; while the direct effect of maturation on the embryo, which results from desiccation, is the same in both varieties.

SUMMARY

1. The germination behaviour of a white and a red wheat variety has been compared at different stages during maturation and has been related to other changes which take place in the grain. Germination tests have also been made

with grains treated in various ways to determine the part played by the embryo and the covering layers at each stage.

2. During development and ripening there was a progressive increase in the rate of development under germination conditions when the embryos were exposed; and a similar rate was achieved when the epidermis of the pericarp only was removed; but in the early stages embryo development was completely inhibited in freshly harvested grains with the covering layers intact.

3. At the harvest-ripe stage the embryos in the white grains were capable of rupturing the covering layers early in the test, whereas emergence of the embryos in the red grains was considerably delayed. Removal of the covering layers eliminated the varietal difference in the rate of embryo development.

4. The delayed germination of the red grains was not due to the impermeability of the covering layers to water, which was rapidly absorbed by the embryos during the initial phase of uptake by imbibition. But it was associated with a retardation of the subsequent uptake, which accompanies cell-vacuolation and growth, until the covering layers had been ruptured.

5. Conditions affecting the supply of oxygen to the embryo did not appear to be a limiting factor as the rate of embryo development was not increased when it was exposed at the micropyle. And, moreover, the rapid water uptake was continued after the embryo had imbibed, when free access of water was provided through the distal end of the grain without affecting the path of gaseous diffusion to the embryo.

6. Premature desiccation caused the disappearance of starch from the embryos and increased their rate of development, when exposed, to that of normally ripened grains at the harvest-ripe stage. There were no varietal differences in the germination of intact grains after pre-desiccation at 3 weeks after anthesis, but the germination of the red grains was increasingly delayed when they were treated at later periods, reaching a minimum at 6 weeks after anthesis. During this period it was observed that thickening was laid down on the walls of the epidermal cells.

7. The germination behaviour of the two varieties appears to be determined by the mechanical properties of the covering layers. In the red grains these impose a greater limitation on the expansion of the embryo, and delay its emergence until there has been an increase in the water-absorbing power of the embryo sufficient to enable it to overcome the constraint.

8. The increased water-absorbing capacity of the embryo may result from an increase in osmotic pressure, either by the transfer of carbohydrate from the endosperm which is facilitated when water is supplied through the distal end of the grain; or by the hydrolysis of the starch already present in the embryo as a result of desiccation during the final stages of ripening and during after-ripening in dry storage.

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The Nitrogen-Nutrition of *Hippophaë rhamnoides* L.

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With Plates XXIII and XXIV and two Figures in the Text

ABSTRACT

It is shown that *Hippophaë* plants, inoculated at the six-leaf stage with a suspension of crushed nodules, begin to develop nodules in about two weeks and soon afterwards become able to grow satisfactorily in culture solution free of combined nitrogen, up to 79 mg. nitrogen per plant having been accumulated during 7 months' active growth. It is concluded that the nodules fix atmospheric nitrogen, and that in its nitrogen nutrition the *Hippophaë* plant shows a close similarity to the nodulated legume. It is pointed out that *Hippophaë* and the other Angiospermous genera with nitrogen-fixing root nodules are all woody plants or belong to a family which includes woody types. The possible significance of this is briefly discussed.

INTRODUCTION

THE genus *Hippophaë*, of which two species are usually recognized, with *H. rhamnoides* L. (Sea Buckthorn) as the more important, is spread over a vast area of Asia and Europe, from the Himalayas to the Arctic Circle, situations near to mountain torrents, rivers, lakes, or the sea being especially typical. In common with the two other genera of the family Elaeagnaceae, namely *Elaeagnus* and *Shepherdia*, *Hippophaë* has the capacity to form nodules in response to invasion by a particular micro-organism, the identity of which has been discussed by a number of workers (see Hawker and Fraymouth, 1951, for references). It is clear from the literature that nodules are frequently and probably typically present on plants in the field. Nodules have been found at five out of six localities for *H. rhamnoides* in Britain, the exception being Tentsmuir, Fife, but it is possible that here nodule-bearing roots have become very deeply buried as the result of the accumulation of sand round the plants.

Servettaz (1909) appears to have been the first to investigate the significance of the root nodules of *Hippophaë*. He inoculated the roots of young plants with a crushed nodule suspension and found that such plants grew much more strongly than uninoculated plants, a sandy rooting medium low in combined nitrogen being employed. Subsequent inspection revealed many nodules on the former plants, but only few on those which were uninoculated. He concluded provisionally that nitrogen fixation had occurred in association with the nodulated plants. Roberg (1934), in a study of cross-inoculability among

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non-legume root nodule plants, reported that he had noted evidence of fixation in nodulated *Hippophaë* plants. Bond, Fletcher, and Ferguson (1954) presented preliminary evidence of fixation, and also referred briefly to the effect of various factors on nodule development in *Hippophaë*. In the present paper these aspects will be examined in more detail, together with others also relating to the nitrogen nutrition of the species.

METHODS

Fruits obtained during winter months from Freshfield, Lancashire, and Tentsmuir, Fife, were allowed to lie in the greenhouse until partially wizened, and the seeds then extracted and stored in slightly moist sand for several weeks at 2° C. They were subsequently sown in trays of sand. The advisability of this cold treatment was established in preliminary trials. In one such trial, seed collected from the Freshfield station in late January, 1952, was employed, and the germination of seed stored at room temperature and of other seed given 7 weeks cold treatment as above was compared. After 17 days from sowing, only 24 seedlings had appeared from 400 seeds previously stored at room temperature, while from two samples of 400 cold-stored seeds 354 and 386 seedlings had developed respectively. Further seedlings appeared later from the room-temperature sample, but after 6 weeks from sowing the number was still only 200. In another trial, with seed collected at Tentsmuir in November, 1952, it was found that 4 weeks cold treatment was as effective as longer periods, and also that storage at —8° C. was less beneficial than that at 2° C.

At a stage when the hypocotyl had become erect but the cotyledons were still unopened, the seedlings were transplanted into water culture in Crone's solution (nitrogen-free formula, minor elements added, pH adjusted to the desired level), 2-litre glass or glazed earthenware jars covered by teak tops with holes for 7 plants being employed. When nodule development was desired the seedlings were normally supplied with 20 mg. of ammonium-nitrogen per litre of culture solution and allowed to grow on for approximately 1 month, by which time 6 leaves were present per plant and the hypocotyl had attained a height of 2·5 cm. The plants were then transferred to nitrogen-free solution and after 2 days were inoculated. For this purpose 3·5 gm. of washed nodules from field plants were pulverized in 100 ml. water, and the root systems of the cultured plants dipped into the supernatant fluid. Without this inoculation the plants did not develop nodules. Data will be presented later showing that inoculation immediately after the original transplanting did not result in satisfactory nodulation. The field nodules employed for inoculation were obtained from the grounds of the Gatty Marine Laboratory, St. Andrews, from Freshfield, and from Dirleton, East Lothian.

The plant cultures were placed in the greenhouse; no forced aeration was provided. Where necessary the pH of the culture solution in the jars was tested daily and suitably corrected if drift had occurred. The culture solution was renewed at intervals of 3 to 4 weeks.

DATA OBTAINED

1. *Growth of plants on combined nitrogen*

It is well known that although under natural conditions of growth the typical legume is nodulated and utilizes at least a proportion of nodule nitrogen, the plant will grow satisfactorily without nodules on externally supplied combined nitrogen alone. The ability of *Hippophaë* to do likewise has been examined.

For this purpose substantial amounts of combined nitrogen in the form of ammonium sulphate or sodium nitrate were supplied to the young plants immediately after they had been set up in water culture, and further amounts added periodically. The plants were not inoculated and remained free of nodules. Five pH levels were employed, and at the lowest (pH 3·2) little growth occurred and after a short time the plants died. At higher pH levels vigorous growth occurred. The plants were harvested after 20 weeks' growth, the data obtained being shown in Table I, while photographs are provided in Plate XXIII, Figs. 1, 2, and 3.

TABLE I

*Harvest Data (Means per Plant) for Non-nodulated Plants supplied with Combined Nitrogen**

pH of culture solution	Ammonium-nitrogen: Height of shoot (cm.)	Ammonium-nitrogen: Dry weight of plant (g.)	Nitrate-nitrogen: Height of shoot (cm.)	Nitrate-nitrogen: Dry weight of plant (g.)
4·2	30·9	0·90	26·1	0·66
5·4	41·2	1·51	39·7	1·33
6·2	43·5	1·81	45·7	1·78
7·0	43·3	1·75	41·6	1·59
8·0	38·1	1·25	36·1	1·17

* Growth period in water culture was from 27 April to 14 September, with 12 plants per treatment, the total combined nitrogen supplied being 125 mg. per plant. The pH indicated is the initial value and that to which it was restored each day. The difference between mean dry weights required for significance ($P = 0·05$), calculated from an analysis of variance, is 0·43 g.

On the statistical basis (see footnote to Table) the relative success of growth, as judged by the dry weight attained, on the two forms of combined nitrogen at different pH levels was as follows:

Ammonium-nitrogen: pH $4·2 < 5·4 = 6·2 = 7·0 > 8·0$
Nitrate-nitrogen: pH $4·2 < 5·4 < 6·2 = 7·0 = 8·0$

Thus the optimal pH region for ammonium-nitrogen is probably somewhat lower than for nitrate-nitrogen. On the whole growth was slightly better with ammonium than with nitrate-nitrogen.

2. *Nodule development*

As noted already, seedlings were customarily transferred to water culture at a stage when the only foliar structures present were the cotyledons. That inoculation of the seedlings at this stage does not result in satisfactory nodulation has been confirmed in repeated tests. Thus in one typical case, only 6 out of 36 such inoculated seedlings eventually developed nodules, one of the few nodulated plants so obtained being shown in Fig. 1A. It appears that plumular development must precede infection and nodule formation, and also that the organism tends not to survive for very long in the vicinity of the roots.

It was in view of the above finding that the practice was adopted of allowing the transplanted seedlings to grow on to the six-leaf stage, with the help of ammonium-nitrogen, prior to inoculation. In most experiments the inoculation of such plants was followed by a first appearance of nodules 14 to 18 days afterwards. The further progress of nodulation in one population of plants is shown in Fig. 2 (Graph A), from which it will be seen that a majority of plants were nodulated after 5 weeks. In a contemporary experiment with seed of different origin and of lower mean weight, nodulation was slower (Graph B). Fig. 1B shows a typical plant with nodules, after inoculation at the six-leaf stage.

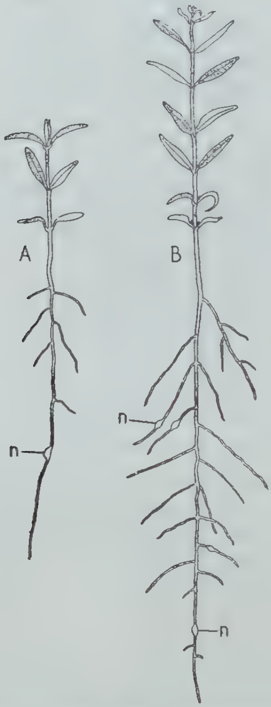

FIG. 1. Plants of *H. rhamnoides* showing root nodules (*n*). A, plant drawn 25 days after inoculation at the cotyledon stage. B, plant 23 days after inoculation at the six-leaf stage. (×½.)

Inocula from different sources varied in infective power on a given occasion. Thus in one comparison in which large numbers of plants raised from Freshfield seed were used, 7 weeks after inoculation with Freshfield inoculum 87 per cent. of the plants were nodulated, whereas this was true of only 52 per cent. of plants inoculated with Dirleton inoculum. A local adaptation between symbionts does not seem to be involved, since on other occasions St. Andrews and Dirleton inocula gave satisfactory nodulation on plants from Freshfield seed.

There was considerable variation between different experiments in the type of nodulation obtained. Frequently the number of initial points of nodulation was small, of the order of 3 or 4 per plant, and subsequent nodule development consisted in the further growth and branching of these original nodules (Pl. XXIII, Fig. 4). In a second type nodules were considerably more numerous, attaining 20 to 30 per plant a few weeks after inoculation (Pl. XXIII, Fig. 5). In a third type nodules were extremely numerous, giving a beaded appearance to the roots, as seen in Pl. XXIV, Fig. 6; a similar condition is sometimes found in leguminous plants. The type of nodulation did not seem to be dependent on the source of the inoculum. Possibly it is determined by seasonal changes in the condition of the endophyte within the nodules used for inoculum preparation.

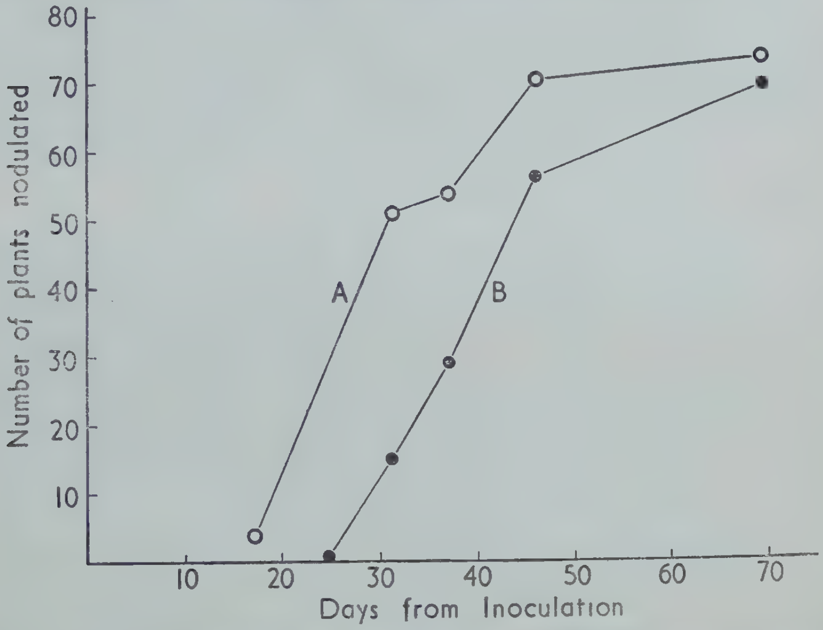

FIG. 2. Progress of nodulation in two populations each comprising 84 plants. Population A was from Freshfield seed, B from Tentsmuir seed. All plants were inoculated on the same date with St. Andrews inoculum.

It is possible that the use of a stronger inoculum than that normally employed (p. 504) would be advantageous. Thus in a recent test, besides the normal strength, inocula of double and half-strength were employed. Seven weeks after inoculation, out of 28 plants at each treatment, 27 bore nodules where the strongest inoculum had been used, and 23 in the case of the two other strengths. In another comparison the use of an inoculum of one-tenth the normal strength resulted in a marked reduction in nodulation.

The pH of the culture solution has an important influence on infection. Bond, Fletcher, and Ferguson (1954) reported that while nodulation was satisfactory and rather similar in extent at pH 7·0, 6·3, and 5·4, none occurred at pH 4·2 or 3·3, although non-nodulated plants supplied with combined nitrogen show considerable growth at pH 4·2, as reported in the present paper. Further experience shows that nodulation is very sparse at pH 5·0, but that occasional nodules form there and also at pH 4·2.

The very potent deterring influence of the presence of combined nitrogen on nodule formation has also been reported previously (Bond *et al.*, loc. cit.). This has been confirmed in further experiments. Thus while in nitrogen-free solution, 20 out of 24 inoculated plants showed nodules 6 weeks after inoculation, in the presence of 5 mg. ammonium-nitrogen per litre of culture solution only 2 plants out of a similar total had nodulated. No nodules developed in the presence of 20 or 50 mg. nitrogen per litre.

3. *The subsequent growth of nodulated plants. Evidence for fixation of nitrogen*

The further growth of some hundreds of nodulated plants in nitrogen-free solution has been studied during the course of the present work, in most cases only to the end of the first season of development, though some plants have been carried over the winter into a second season. Except in the small proportion of cases in which root-browning occurred (see below), the finding has always been that soon after nodule development the plants resume active growth, and the symptoms of nitrogen deficiency which develop prior to nodulation to a large extent disappear, although they persist in control plants, uninoculated and without nodules, and in inoculated plants which fail to nodulate. Pl. XXIV, Fig. 7 shows nodulated and control plants some weeks after inoculation. Data illustrating the superior growth of nodulated plants are provided below in connexion with the study of the effect of pH on growth. Particularly rapid growth was obtained in an experiment in which the second type of nodulation (p. 507) was present. In this, 5 nodulated plants, harvested from a considerable number of similar plants 9 weeks after inoculation, showed a mean shoot height of 21 cm., a mean dry weight of 244 mg., and a mean nitrogen content of 6·9 mg. per plant, the corresponding values for control plants being 5 cm., 55 mg., and 0·5 mg. respectively.

Five further nodulated plants, similar at the end of their first season of growth to those just described, were carried over into a second season. All continued to show substantial growth and further accumulation of nitrogen, as indicated in Table II, the mean nitrogen content at harvest being 60 mg. per plant. The shoot system of one of the plants is shown in Pl. XXIV, Fig. 8. Control plants could not be maintained over the winter, but new controls set up under exactly the same conditions as those provided for the plants in Table II showed the usual minimal growth. The mean percentage nitrogen of the nodule dry matter of plants Nos. 2 and 4 was 6·2, and of the roots and shoots combined was 2·0. The great length attained by the tap-root of three of the plants is notable.

The ability thus demonstrated of nodulated plants to grow satisfactorily and to accumulate nitrogen although supplied with a solution devoid of combined nitrogen indicates that fixation of atmospheric nitrogen must be associated with the plants. At the same time, the growth of nodulated plants has always been considerably less vigorous than that of non-nodulated plants supplied with combined nitrogen. Thus note has already been taken of nodulated plants which attained a mean dry weight of 244 mg. 9 weeks after inoculation; corresponding uninoculated plants supplied with an ample amount of combined nitrogen attained a dry weight of nearly three times that of the nodulated plants, in the same time, but it must be borne in mind that in the case of the nodulated plants about a month elapsed before fixation commenced and growth became possible. Pl. XXIV, Fig. 9 compares the two types of plant grown in a similar experiment.

TABLE II

*Harvest Data for Nodulated Plants grown in Nitrogen-free Culture Solution for Two Seasons**

Plant No.	Height of main shoot (cm.)	Length of tap-root (cm.)	Dry weight (g.)			N content of whole plant (mg.)
			Shoot and root	Nodules	Whole plant	
1	40	45	—	—	2·88	45
2	36	104	2·97	0·08	3·05	64
3	32	40	—	—	2·88	45
4	40	120	3·07	0·09	3·16	65
5	50	130	—	—	5·10	80

* The plants were inoculated 27 July and harvested 24 September of the following year, representing about 7 months of active growth. The pH of the culture solution was approximately 6·0. The main shoots bore 5 to 8 axillary shoots. Some of the plants were combined for Kjeldahl analysis.

TABLE III

*Effect of pH on Growth of Nodulated Plants in Nitrogen-free Solution**

	pH of culture solution	No. of plants	Mean height of shoot (cm.)	Mean dry wt. of plant (mg.)	Mean N content of plant (mg.)
1952	5·4	41	7·7	68	—
	6·3	51	8·3	87	—
1953	6·0	37	12·6	152	2·9
	7·0	25	11·6	164	3·1

* In 1952 the plants were harvested 9 weeks and in 1953 15 weeks after inoculation. Control plants, non-nodulated, showed similar growth in both years and at all pH levels, the mean data based on 37 plants being as follows: height of shoot 5·9 cm., dry weight of plant 54 mg., nitrogen content of plant (1953) 0·5 mg.

The question of the most favourable pH for the growth of nodulated plants has been examined in two separate experiments, detailed in Table III. In the 1952 comparison, growth at pH 6·3 appeared to be rather better than at pH 5·4, but a 't' test on the data for shoot height (individual figures for dry weight were not determined) shows that the difference in the means is not significant ($P = 0{\cdot}05$). The same applies to the 1953 comparison. Thus so far as the present data go, the pH levels 5·4, 6·3, and 7·0 are all favourable to growth.

The effect of the oxygen-supply provided to the root system on the growth of the nodulated *Hippophaë* plant has been studied by the technique of Ferguson and Bond (1954). Already-nodulated plants were sealed into large test-tubes containing nitrogen-free culture solution, and different oxygen/nitrogen mixtures bubbled through the solution for a period of 7 weeks. The effects on growth were as indicated in Table IV. The increases in height, dry weight, and nitrogen content shown over the period for the most part become progressively smaller as the oxygen-supply is diminished. The loss of nitrogen at the two lower oxygen levels reflects the falling of the basal leaves of these plants, preceded by yellowing. Analysis of variance confirms that the over-all effect of oxygen is significant, although only the larger of the differences between means for height or dry weight increments at adjacent oxygen levels attain significance. Typical plants from the experiment are shown in Pl. XXIV, Fig. 10. It is concluded that the nodulated *Hippophaë* plant requires good oxygenation of the rooting medium for maximum growth.

TABLE IV

*Effect of Oxygen Supply to Root System on Growth of Nodulated Plants**

	Mean increase during period		
% oxygen supplied	In height of shoot (mm.)	In dry wt. of plant (mg.)	In N content of plant (mg.)
21	38	67	2·1
10	21	51	1·0
5	18	15	−0·1
1	12	21	−0·4

* The duration of the gas-flow was 7 weeks, with 6 plants per treatment, pH 6·3. The initial heights of the shoots were of the order of 85 mm. Mean initial values of 91 mg. for dry weight and 1·7 mg. for nitrogen content, based on the examination of similar plants, were used in the calculation of the increases in respect of these attributes.

A difficulty that has been experienced with the culture of nodulated plants is that a proportion, usually a small one, of the plants develop a condition of root-browning, followed by a cessation of growth. In most though not all instances these symptoms develop fairly soon after inoculation, and it is possible that they are due to the presence of substances harmful to the roots in the inoculum. Another possible explanation which has been considered is that forced aeration of the culture solution is necessary to secure uniformly good growth of nodulated plants. The sensitiveness to oxygen-supply revealed by

Table IV is not notably greater than in a corresponding experiment with *Alnus*. The latter grows vigorously in unaerated jar culture, but under these conditions *Hippophaë* may be at a disadvantage since the nodules are more deeply submerged owing to the later stage at which the plants are inoculated. However, in a trial the provision of forced aeration did not eliminate root-browning completely, and at present inoculum toxicity appears the most likely explanation.

DISCUSSION

The observations presented above show that the nodulated *Hippophaë* plant, like such plants of *Alnus* and *Myrica*, presents a close approach to the nodulated legume in its relation to nitrogen. The nodules are similar to those of legumes in their physiological significance and relation to the rest of the plant. The following points of particular resemblance to the legume symbiosis may be noted:

(1) under favourable conditions the development of nodules commences on young plants 2 weeks after an application of the nodule organism to the roots;

(2) after nodules have appeared the plant becomes able to grow satisfactorily in a rooting medium free of combined nitrogen, indicating that fixation of atmospheric nitrogen occurs in the nodules, and that a large part of the fixed nitrogen passes into the rest of the plant;

(3) the development of nodules and the derivation of fixed nitrogen from them are not obligate to the growth of the plant, provided an external source of combined nitrogen is available;

(4) the formation of nodules consequent on inoculation of the plant is much reduced in the presence of combined nitrogen in the rooting medium.

It is true that the plants in the present work were not grown under aseptic conditions. It might be suggested that the observed fixation of nitrogen was due, not to a nodule-inhabiting organism, but to some other organism, introduced with the inoculum, which established itself in the rhizosphere, the nitrogen which it fixed becoming by some means available to the *Hippophaë* plant. There is, however, convincing evidence from the present experiments and from parallel ones with other non-legumes (Ferguson and Bond, 1953; Bond 1955) that the fixation is specifically a nodular function. But it is not yet proved that in the non-legume nodule there is a simple symbiosis of a single endophyte and the higher plant cells, though the probability of this is increased by Quispel's demonstration (1954) that in *Alnus* fixation was still shown by plants the nodules of which included no micro-organisms cultivable separately on standard media.

The present evidence relates only to fixation by the nodules developing on cultured plants as a result of artificial inoculation, but it must be regarded as extremely probable that the nodules on field plants likewise have the ability to fix nitrogen. In the case of *Alnus*, Bond (1956) has confirmed the implications of greenhouse experiments by demonstrating, by isotopic technique, that the nodules of trees in the field fix nitrogen. The provisional conclusion may be reached that *Hippophaë* regularly derives a supplementary source of combined nitrogen from its nodules, the degree of supplementation being probably inversely related to the abundance of available combined nitrogen in the soil.

The differences existing between *Hippophaë* and the two other non-legume root nodule plants for which comparable data exist, namely *Alnus* and *Myrica*, are chiefly in respect of the pH relation. Thus the optimum pH for the growth of nodulated plants of *Hippophaë* probably lies in the range 6·0 to 7·0, compared with a figure of slightly less than pH 5·0 for *Alnus* and 5·4 for *Myrica* (Bond, Fletcher, and Ferguson, 1954). The *Hippophaë* nodule organism, at least in respect of its infective capacity, is markedly less tolerant of acid conditions, since a pH of 5·0 results in almost complete suppression of nodule formation, while extensive nodulation occurs in *Alnus* and *Myrica* at considerably lower pH levels.

It is of interest that the three genera (*Elaeagnus*, *Hippophaë*, and *Shepherdia*) that comprise the family Elaeagnaceae all form root nodules. Willis (1948) gives 30 species for *Elaeagnus*, occurring chiefly in southern Europe and Asia, but with species also in Australia and North America. *Shepherdia* has three species, all North American. If single genus families are excluded, then it is probable that in the Elaeagnaceae we have the only Angiospermous family all of whose members form root nodules. Allen and Allen (1954) have emphasized that ability to nodulate does not characterize all legumes. According to those authors, records regarding ability to form nodules are available for only some 1,200 of the 10,000 species included in the family, but already it appears that about 100 species, in 20 genera, are unable to form nodules.

Plants forming root-nodules are widely scattered in the Angiosperms. The following is a list of the generally recognized instances, the systematic position indicated being according to Engler and Diels (1936):

Genus	Family	Order
Hippophaë *Shepherdia* *Elaeagnus*	Elaeagnaceae	Myrtiflorae
Alnus	Betulaceae	Fagales
Myrica	Myricaceae	Myricales
Coriaria	Coriariaceae	Sapindales
Casuarina	Casuarinaceae	Verticillatae
Ceanothus	Rhamnaceae	Rhamnales
Many genera	Leguminosae	Rosales

Myrica, *Coriaria*, and *Casuarina* are each the only genus in their families. Although no two of the families listed lie in the same order, it can at least be stated that they are all in the Archichlamydeae, and further that no instance of root nodule formation in a Monocotyledonous plant is known to the authors. Examples do occur in the Gymnosperms, but for the present they will be excluded from consideration since the activities of such nodules have received little attention, whereas there is strong evidence in the literature that the nodules of the Angiospermous genera listed above are nitrogen-fixing. It is of interest that all the non-legumes listed are woody plants, and in fact the general statement can be made that all accepted instances of root-nodule formation involve woody plants, or, as in the legumes, plants in a family which includes woody genera. This is probably not without significance. If the widely held theory that the first flowering plants were of woody nature is accepted, then it seems likely that the possession of root nodules and the ability so to use atmospheric nitrogen are very ancient characters, and mostly date from a time when only woody flowering plants existed and when conditions were apparently particularly favourable to the initiation of symbiotic associations. These aspects will be discussed further in a later publication.

SUMMARY

The significance in nitrogen nutrition of the root nodules of *Hippophaë* has been investigated. Nodules begin to appear in about two weeks' time when young plants in water culture at the six-leaf stage are inoculated with a suspension of crushed nodules from other *Hippophaë* plants. The type of nodulation obtained in different experiments has varied in respect of number and size of nodules, possibly because of seasonal variation in the condition of the endophyte in the nodules used for inoculation. Nodulation is satisfactory over the pH range 7·0 to 5·4, but not at lower levels. The presence of combined nitrogen in the rooting medium greatly reduces nodule formation.

After nodules have formed the plant becomes able to make satisfactory growth in a culture solution free of combined nitrogen, and it is concluded that the nodules are the centres of a symbiotic fixation of atmospheric nitrogen. A fixation of up to 7·5 mg. nitrogen per plant has been observed during the 9 weeks following inoculation, and of up to 79 mg. during two seasons' growth. *Hippophaë* grows vigorously without nodules if supplied with ammonium or nitrate nitrogen under suitable conditions of pH. It is concluded that in its nitrogen nutrition *Hippophaë* resembles the legume.

It is pointed out that all the recognized root nodule forming plants in the Angiosperms are of woody type or belong to a family which includes woody genera. It seems likely that the possession of root nodules and the ability so to use atmospheric nitrogen are very ancient characters, and mostly date from a time when only woody flowering plants existed.

The authors are indebted to Dr. L. W. Poel and Mr. D. A. Coult for the provision of *Hippophaë* fruits and nodules, and to Mr. W. Anderson and Miss K. McCormick for photographic and technical assistance respectively.

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EXPLANATION OF PLATES

Illustrating the article by G. Bond, J. T. MacConnell, and A. H. McCallum on 'The Nitrogen-Nutrition of *Hippophaë rhamnoides* L.'

PLATE XXIII

Fig. 1. Non-nodulated plants grown in water culture for 20 weeks with ammonium sulphate supplied, at pH (left to right) 4·2, 5·4, 6·2, and 7·0 respectively. (× 1/12.)

Fig. 2. As Fig. 1, except that sodium nitrate was supplied instead of ammonium sulphate.

Fig. 3. Single plants grown at pH 7·0 from the experiment to which Fig. 1 refers. (× 1/10.)

Fig. 4. Root system of nodulated plant, showing sparse type of nodulation. (× 2/3.)

Fig. 5. Plant, 5 weeks after inoculation, showing fairly abundant nodules. (× 7/16.)

PLATE XXIV

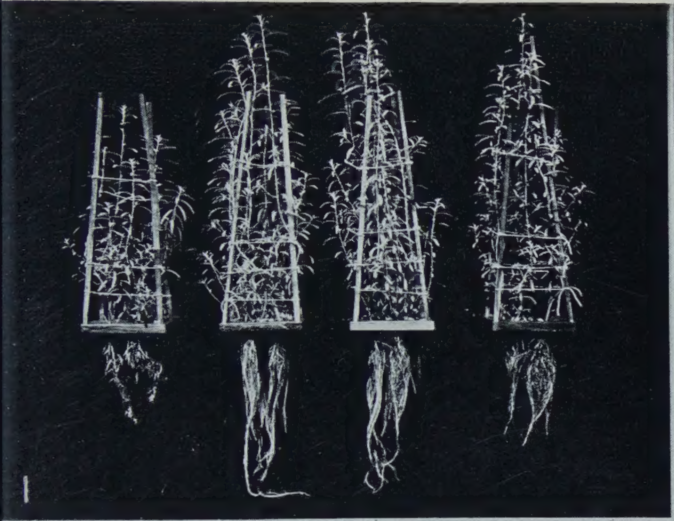
Fig. 6. Part of root system of plant with very numerous nodules, giving beaded appearance. (× 7/16.)

Fig. 7. Nodulated (left) and non-nodulated plants grown in solution free of combined nitrogen, 14 weeks after inoculation. (× 1/5.)

Fig. 8. Shoot of nodulated plant, inoculated at the six-leaf stage in July and grown in solution free of combined nitrogen until August of the following year. (× 1/6.)

Fig. 9. Nodulated plant (left) grown in nitrogen-free solution for 14 weeks following inoculation, and non-nodulated plant grown for same period in presence of 100 mg. ammonium-nitrogen per litre of culture solution, both at pH 6·3. (× 1/8.)

Fig. 10. Nodulated plants grown for 7 weeks with oxygen/nitrogen mixtures containing 1 per cent. (left) and 21 per cent. (right) oxygen supplied to the root systems. (× 1/4.)



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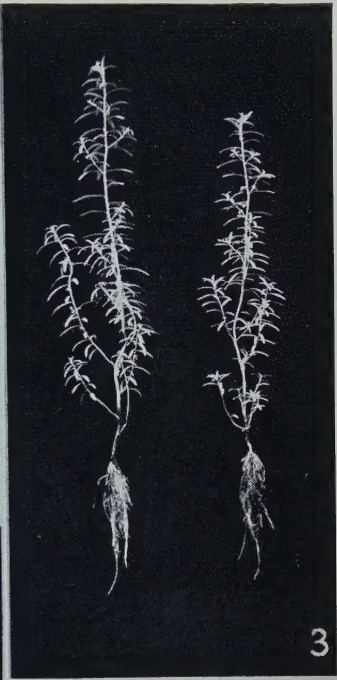

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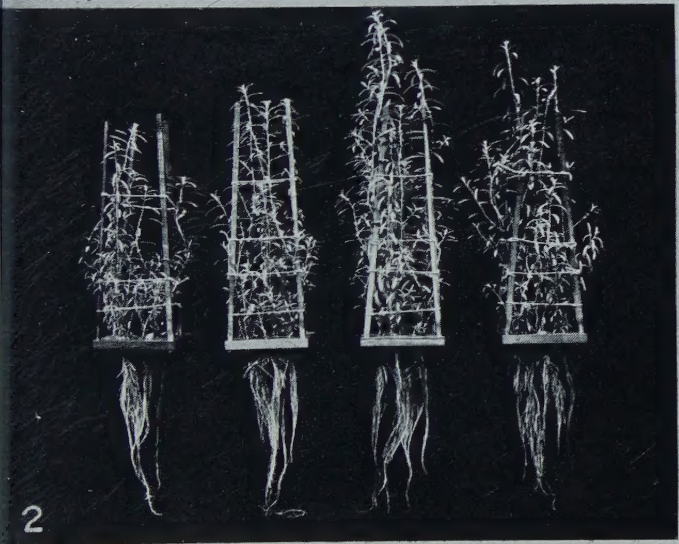

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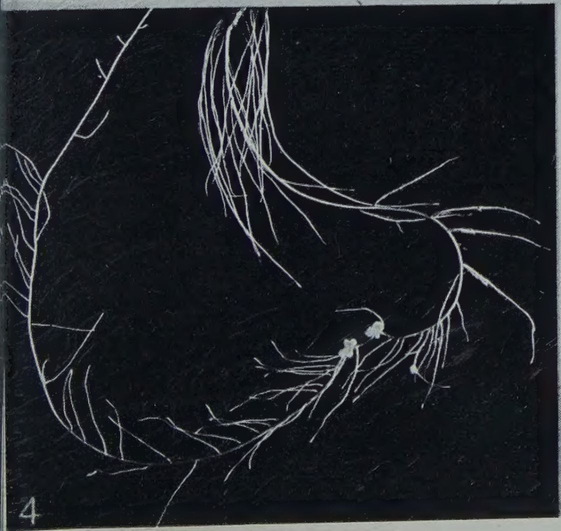

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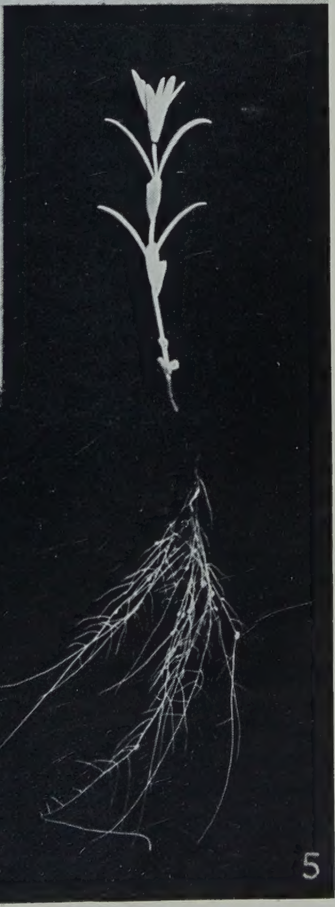

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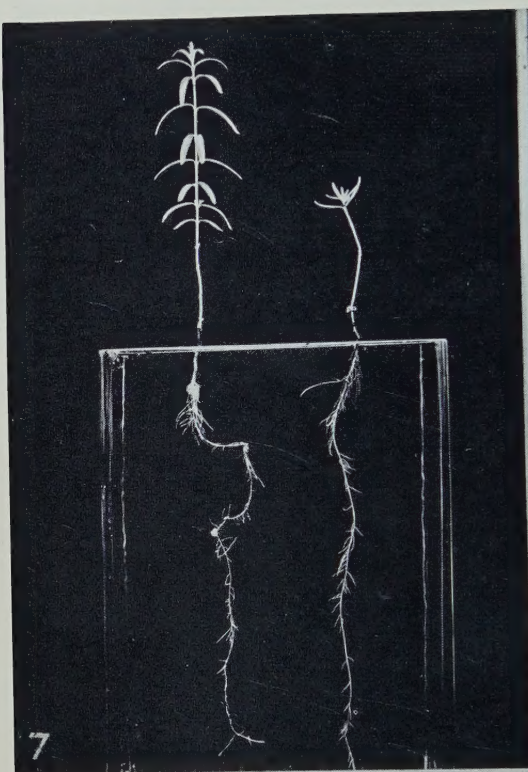
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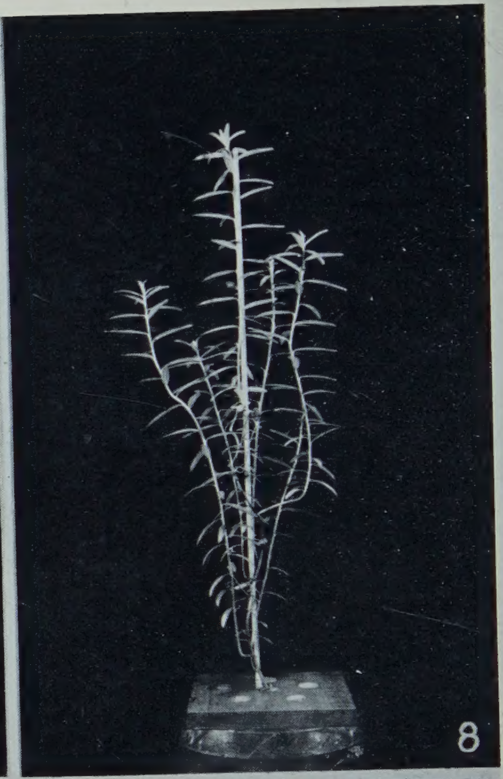
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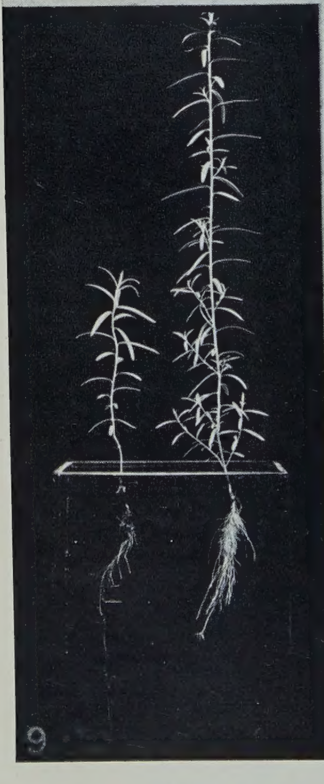
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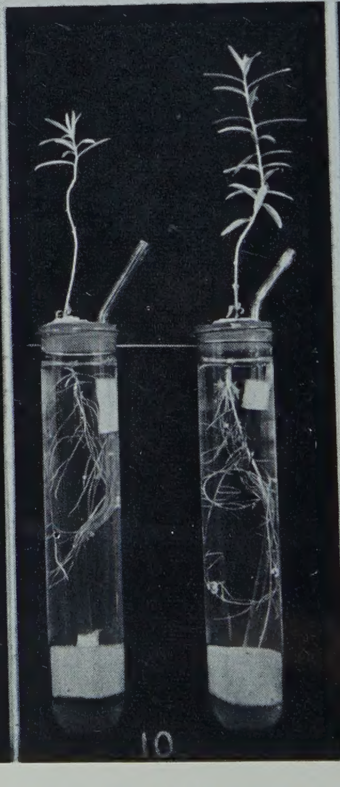
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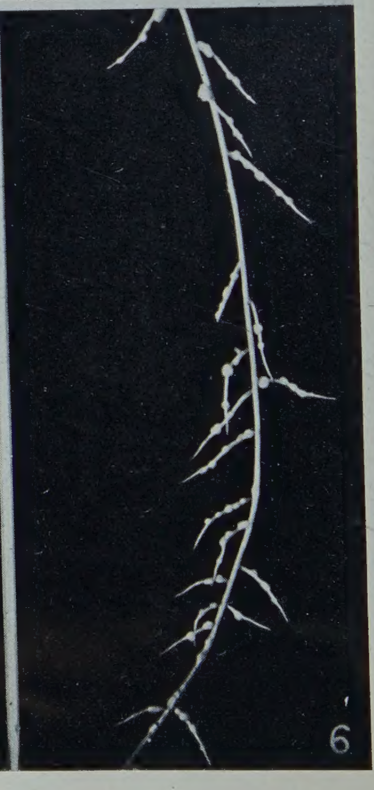
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